Regional Distribution, Developmental Changes, and Cellular Localization of CNTF–mRNA and Protein in the Rat Brain

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Abstract. Ciliary neurotrophic factor (CNTF) is a potent survival molecule for a variety of embryonic neurons in culture. The developmental expression of CNTF occurs clearly after the time period of the physiological cell death of CNTF-responsive neurons. This, together with the sites of expression, excludes CNTF as a target-derived neuronal survival factor, at least in rodents. However, CNTF also participates in the induction of type 2 astrocyte differentiation in vitro. Here we demonstrate that the time course of the expression of CNTF–mRNA and protein in the rat optic nerve (as evaluated by quantitative Northern blot analysis and biological activity, respectively) is compatible with such a glial differentiation function of CNTF in vivo. We also show that the type 2 astrocyte–inducing activity previously demonstrated in optic nerve extract can be precipitated by an antiserum against CNTF. Immunohistochemical analysis of astrocytes in vitro and in vivo demonstrates that the expression of CNTF is confined to a subpopulation of type 1 astrocytes. The olfactory bulb of adult rats has comparably high levels of CNTF to the optic nerve, and here again, CNTF-immunoreactivity is localized in a subpopulation of astrocytes. However, the postnatal expression of CNTF in the olfactory bulb occurs later than in the optic nerve. In other brain regions both CNTF–mRNA and protein levels are much lower.

C ILIARY neurotrophic factor (CNTF)[1] was originally identified (Helfand et al., 1976) and partially purified (Barbin et al., 1984) as a target-derived neurotrophic molecule supporting the survival of parasympathetic chick ciliary neurons. It rapidly became apparent that the spectrum of biological activities of CNTF is much broader, in that it also supports the survival of sympathetic and sensory neurons (Barbin et al., 1984). More recently CNTF was shown to support the survival of chick motoneurons (Arakawa et al., 1990), and its local administration to early postnatal rats was shown to prevent the lesion-mediated degeneration of motoneurons (Sendtner et al., 1990). In addition to these neurotrophic activities, CNTF was demonstrated to have specific differentiating properties. In primary cultures of newborn rat sympathetic neurons, CNTF induces cholinergic properties reflected by an increase of choline acetyltransferase activity (ChAT) and, reciprocally, reduction of tyrosine hydroxylase activity, the rate-limiting enzyme in the synthesis of the adrenergic transmitter norepinephrine (Saadat et al., 1989). In cultures of newborn rat optic nerve cells, CNTF initiates the differentiation of O-2A progenitor cells to type 2 astrocytes (Hughes et al., 1988; Lillien et al., 1988).

The in vitro differentiation of O-2A progenitor cells into oligodendrocytes and type 2 astrocytes has been investigated in great detail (Lillien and Raff, 1990). One aim of the present investigation was to evaluate whether the time course of expression of CNTF mRNA and CNTF protein in vivo is correlated with the time of appearance of the first cells with the antigenic phenotype of type 2 astrocytes in cultures of newborn rat optic nerve. A second aim was to determine whether type 1 astrocytes synthesize CNTF in vivo as they do in vitro. Finally, we wanted to compare the time course and level of CNTF expression in the optic nerve to that in other regions of the central nervous system (CNS) and to that previously determined in the sciatic nerve (Stöckli et al., 1989). We show that the changes in CNTF protein and mRNA levels in the optic nerve correspond to the time course predicted by studies on the timing of type 2 astrocyte development in vitro, with both mRNA and protein reaching maximal levels in the second postnatal week. CNTF protein and mRNA reached comparably high levels in the olfactory bulb, but were substantially lower in other brain areas.

Materials and Methods

RNA Preparation and Northern Blot Analysis

Total RNA of different tissues was isolated and processed for Northern blot

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1. *Abbreviations used in this paper*: ChAT, choline acetyltransferase; CNS, central nervous system; CNTF, ciliary neurotrophic factor; PCR, polymerase chain reaction.

analysis as described (Chomczynski et al., 1987). 20 picograms of a short-ened synthetic 0.6-kb CNTF–RNA standard corresponding to the coding re-gion of CNTF was added to each tissue sample as a recovery standard before RNA extraction. Following electrophoresis through a 1.4% agarose gel con-taining 2.0 M formaldehyde (Lehrach et al., 1977), RNA was vacuum blot-ted to nylon membranes (Hybond-N; Amersham Corp., Arlington Heights, IL). CNTF standards (0.6 and 0.34 kb) were coelectrophoresed in separate lanes. This, together with the determination of the recovery in the individual samples (Heumann et al., 1986), permitted the determination of the abso-lute CNTF mRNA values.

Hybridization was performed overnight at 65°C in 10 ml hybridization buffer (5 × SSC, 50% formamide, 5 × Denhardt's, 20 mM Na_xPO_4, pH 7.0, 5 mM EDTA, 1% SDS, 350 μg/ml denatured salmon sperm DNA) with a [^{32}P]-cRNA rat CNTF probe (5 × 10^6 cpm/ml). After washing in 0.1 × SSC containing 0.5% SDS at 70°C, the blots were exposed for 24 h to an X-ray film (Fuji) at −70°C with intensifying screens.

After autoradiography the films were densitometrically scanned and the CNTF mRNA values were corrected according to the individual recoveries. The CNTF mRNA values were expressed in picogram/μg of total RNA, or in percentage of sciatic nerve mRNA levels, given as the mean ± SEM.

The cRNA probes were prepared from a Bluescript SK + vector contain-ing the entire coding region of the rat CNTF–cDNA (Stöckli et al., 1989). The vector was linearized with EcoRI and a single-stranded RNA probe was transcribed using the Promega in vitro transcription system with T3 RNA polymerase. The specific activity of this probe was 10^9 cpm/μg.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) was performed with AmpliTaq Poly-merase from Perkin-Elmer Corp. (Ueberlingen, Germany) (N801-0046) ac-cording to the manufacturer's recommendation, using the Perkin-Elmer DNA Amplification Kit (N801-0055) and the Perkin-Elmer Thermal Cycler (Perkin-Elmer N801-0177). For amplification of a 600-bp rat CNTF-cDNA fragment oligonucleotide primers covering the first 20 bp of both the very 5′ end (primer 1: ATGGCTTTCGCAGAGCAAACA) and the very 3′ end (primer 2: CATCTGCTTATCTTTGGCCCC) of the coding region of rat CNTF–cDNA (Stöckli et al., 1989) were used. Briefly, 20 μg of total RNA from embryonic tissues were transcribed according to the manufacturer's recommendation using the superscript M-MLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) and primer 2 as a specific primer of cDNA synthesis. 10 μl of the single strand cDNA obtained was used after additional treatment with 20 U of DNAse I (FPLCpure, Pharmacia Fine Chemicals, Heidelberg, Germany, NJ; 15 min, 37°C) for a 100 μl PCR reaction containing the following components: 10 μl of 10 × TaqBuffer (1 × TaqBuffer is 10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatine), 5 μl of dNTP's (each 5 mM), 3 μl of each primer oligonucleotide (each 50 μM), 1 μl (5 U/μl) of Taq Pol, with water to a final volume of 100 μl. The components were carefully mixed by vortex-ing and briefly spun before the initiation of the PCR reaction. To reduce evaporation of refluxing, the reaction mix was overlayed with two drops of mineral oil (Perkin-Elmer No. 0186-2302). Optimal temperature used for this PCR reaction was shown to be 60°C. The expected molecular size of the product thus amplified was 600 bp.

Cell Culture

Primary brain cultures from newborn rats were prepared according to McCarthy et al. (1980). The cells were kept in DME supplemented with 10% FCS, 100 μg/ml streptomycin, 100 U/ml penicillin, and grown in 10% CO_2/90% air at 37°C. The cells were confluent after 2 wk. Following two to three passages (4 wk in culture) the cells were lysed for Western blotting or fixed for immunohistochemistry. Astrocyte cultures from newborn rat brain and Schwann cell cultures from newborn sciatic nerve were prepared as described by Spranger et al. (1990) (astrocytes) and Matsuoka et al. (1991) (Schwann cells).

Bioassay

The chick ciliary neuron survival assay was performed as described previ-ously (Hughes et al., 1988). Briefly, ciliary ganglia were dissected from E8 chick embryos, trypsinized, dissociated, and preplated for the enrichment of neurons. The neurons thus obtained were cultured at a cell density of 500–1,000 cells/well in 24-multi-well dishes (Costar) coated with polyor-nithine and laminin. The medium used was F14 supplemented with 10% horse serum. Tissue extracts were added at five different concentrations and the number of surviving neurons was counted after 24 h in culture. The pro-tein concentration (μg/ml) that supported half-maximal survival of the cul-tured neurons was defined as one trophic unit.

Antisera

Two antisera were raised against synthetic peptides: Petide I (M-V-L-L-E-Q-K-I-P-E-N-E-A-D-G-M-P-A-T-V-G-D-G-G-L-F-E, antiserum 1) corre-sponds to amino acid 127-153 and Petide II (I-S-A-L-E-S-M-Y-G-K-D-K-Q, antiserum 2) corresponds to amino acids 186-199 of the rat CNTF sequence (Stöckli et al., 1989). The peptides were coupled to keyhole limpet hemocyanine as described by Kitagawa and Aikawa (1976) using modifica-tions described by Liu et al. (1979). For GFAP immunohistochemistry, a mouse mAb against GFAP (Boehringer Mannheim Biochemicals, Mann-heim, Germany) (see Figs. 3 and 6 f) and a rabbit antiserum against GFAP (Dako Corp., Santa Barbara, CA) (see Fig. 6 h) were used.
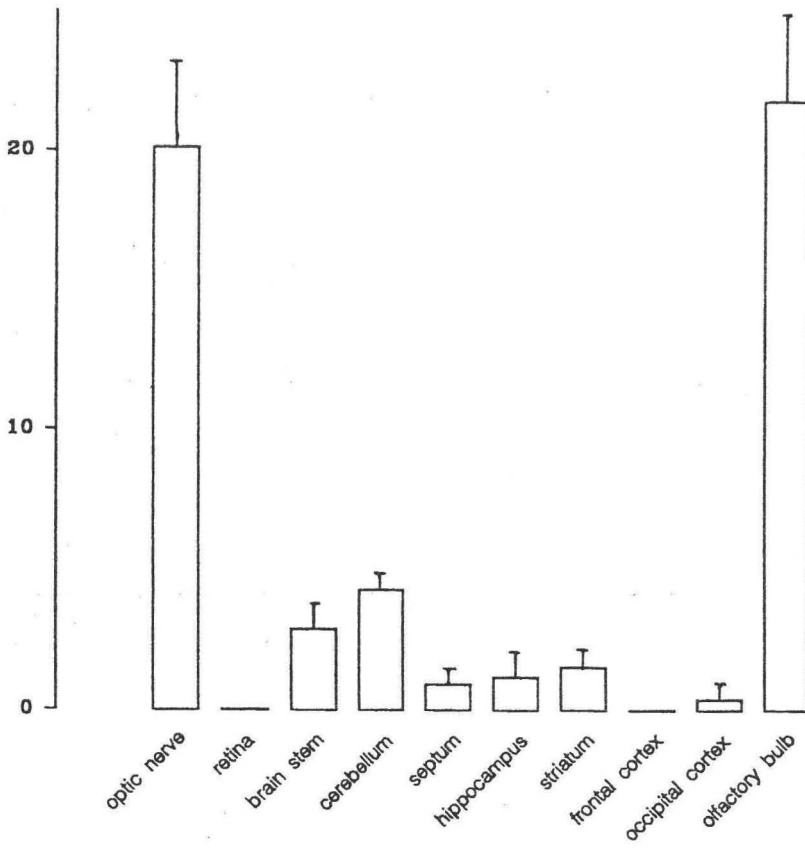
Immunofluorescence

Cultures of newborn rat brains were fixed for 5 min in 4% paraformalde-hyde, preincubated with PBS, 0.1% Triton, and 1% BSA, followed by incu-bation with anti-GFAP (Boehringer Mannheim Biochemicals; 1:50) and a sheep anti-mouse FITC second antibody (Nordic Immunology, Tilburg, The Netherlands; 1:100). Costaining for CNTF was then performed using the rabbit antiserum against peptide I (diluted 1:500), biotinylated donkey anti–rabbit F(ab)$_2$ (Amersham Corp., 1:100) and Texas red–coupled strep-tavidin (Amersham Corp., 1:100). Controls for the specificity of CNTF staining were performed by adding excess peptide I (1 mg/ml) to the incuba-tion with the anti-peptide I antiserum. Short-term cultures (2 h) were pre-pared from 3-wk-old rat olfactory bulb. After freeing the tissues of meninges and incubation with 0.25% Trypsin for 20 min, the tissue was dissociated by passing it five times through a 1.1-mm-diam syringe. Cells were cen-trifuged at 400 g for 5 min, the medium changed to DME plus 10% FCS and plated on polylysine coated Greiner four-well dishes. Double staining for GFAP and CNTF was performed as previously described except that a rabbit anti–GFAP antiserum (Dako Corp., 1:500) and a mouse monoclonal anti–CNTF antibody (4-68, hybridoma supernatant diluted 1:2 in PBS) were used. As second antibodies a goat anti-rabbit FITC antiserum (Nor-dic) and a biotinylated sheep anti–mouse antiserum (Amersham Corp.) were used at the same dilutions as previously described. Controls were per-formed by preincubation of the anti-CNTF hybridoma supernatant with 300 μg/ml of recombinant rat CNTF.

For immunohistochemistry with optic nerve, a 24-d-old rat was anaesthetized, perfused with 4% formaldehyde, and the optic nerve dis-sected. The sciatic nerve and olfactory bulb were removed from an adult rat after perfusion. Tissues were postfixed for 2 h, dehydrated overnight with 30% sucrose, and frozen sections (7 μm) were dried on glass slides previously coated with gelatine, rehydrated with gelatine buffer (0.1 M Tris/PO$_4$, pH 7.0 containing 0.1% gelatine, and 0.2% Triton), and incubated in the same buffer with antiserum (1:200) against peptide I. Controls were performed by co-incubation with excess peptide I (see Fig. 5) or recombinant CNTF (see Fig. 6) as described above. For olfactory bulb sections, the mouse anti–GFAP antibody and the anti-CNTF mAb were used at the same dilutions as previously described. Following incubation with biotinylated second antibodies and Texas red streptavidin, the sections were embedded

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Figure 1. Distribution of (*a*) CNTF mRNA and (*b*) chick ciliary neuron survival activity in regions of the adult rat CNS. In *a* levels are reported as a percentage of the signal intensity observed in Northern blots with RNA from adult rat sciatic nerve (on average 16 ± 2 pg CNTF mRNA/10 μg of total RNA). In *b* the survival activity for E8 chick ciliary neurons in culture was determined with extracts from various regions of the adult rat CNS. The values shown are presented as trophic units per mg extractable protein. One trophic unit is defined as the activity necessary to support half-maximal survival of the ciliary neurons after 24 h in culture.
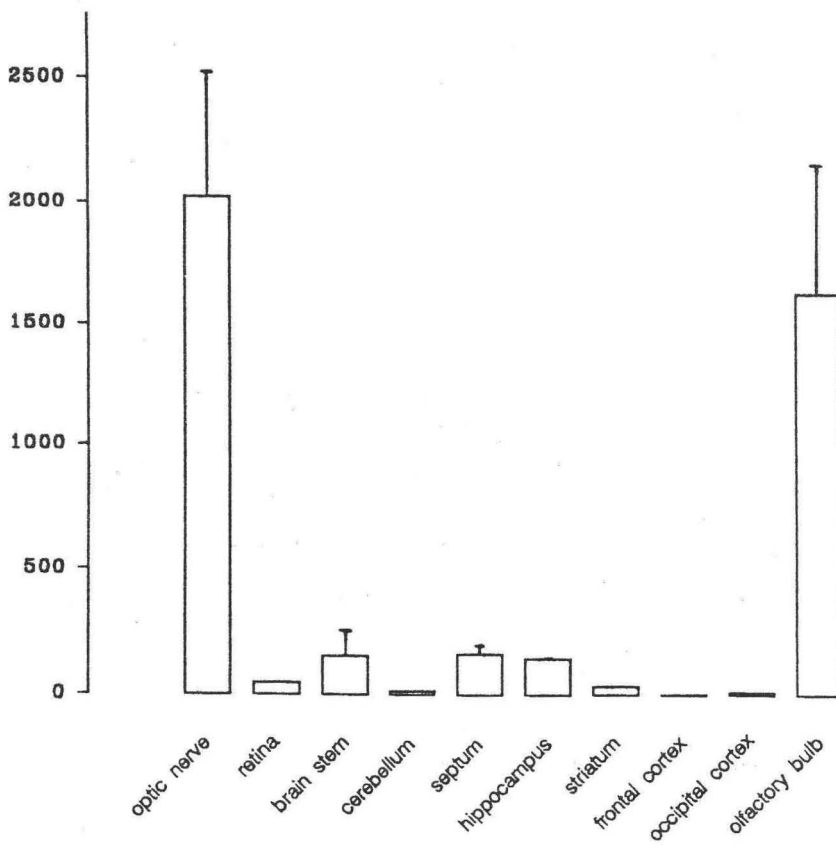
a

b

with 50% PBS/50% glycerol and examined using an Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany).

Preparation of Tissues for Bioassays and Western Blot Analysis

After three washes with PBS, brain cell cultures were incubated with a hypotonic (5 mM) phosphate buffer (pH 7.0) containing 30 mM NaCl, removed from the culture dishes with a rubber policeman and disrupted by 10 cycles of 1-s duration in an ultra sonicator. Following ultra-centrifugation at 100,000 g for 30 min in a TL-100 ultra-centrifuge (Beckman Instruments Inc., Palo Alto, CA), the supernatant was removed. Protein concentrations of the supernatants were determined using the Coomassie blue–based protein assay (Bradford, 1974; Bio Rad). Sciatic nerve and other tissue extracts were prepared after homogenization using a glass–glass homogenizer as described previously (Saadat et al., 1989). For the bioassays the extracts were filtered. For Western blot analysis of sciatic nerve extracts and of brain culture extracts, 50 μg of protein were run per lane on 10–20% polyacrylamide gels under reducing conditions. Recombinant rat CNTF (Squinto et al., 1990) at appropriate concentrations was coelectrophoresed in separate lanes. After blotting on a nitrocellulose membrane (Schleicher and Schüll, Inc., Dassel, Germany) for 90 min at 150 mA using a 2117 Multiphor Blotting apparatus (LKB Instruments, Inc., Bromma, Sweden), the blots were blocked with TBS containing 0.1% Tween and 1% dry milk powder and incubated with anti-peptide I antiserum (1:1000). In Fig. 1 an additional antiserum (1:1,000) against peptide II was used, incubated overnight at 4°C, washed three times, blocked in the buffer described before, and detected using an HRP-coupled goat anti-rabbit antiserum (Nordic Immunology, Tilburg, The Netherlands; 1:500). The stained bands were visualized with chloronapthol.

Immunoprecipitation

15 mg of Prot-A-Sepharose (Pharmacia Fine Chemicals) were suspended in 100 μl of TBS buffer (10 mM Tris/150 mM NaCl, pH 8.0) per tube and 200 μl of anti-peptide II antiserum was added for 1 h. The Sepharose with coupled antibodies was washed three times with 500 μl of TBS by centrifugation and discharging the supernatants. Tissue extracts were added after dilution with TBS in a volume of 400 μl. After centrifugation the supernatants were removed and tested for their ability to induce GFAP in O-2A progenitor cells in cultures of newborn optic nerve cells (Hughes et al., 1988). The extent of immunoprecipitation was determined in separate reactions by adding 200 trophic units of purified rat CNTF (Stöckli et al., 1989). Usually more than 95% of the CNTF was bound to the coupled antibodies and could be removed from the supernatant by centrifugation.

Results

Distribution of CNTF mRNA in the Adult Rat Brain

Using quantitative Northern blot analysis, high levels of CNTF mRNA were found in the optic nerve and olfactory bulb of adult rats; intermediate levels were found in the cerebellum and brain stem while low levels were found in other regions, such as hippocampus, striatum, cortex, and septum (Fig. 1 a). No CNTF mRNA could be detected in the retina. The distribution of CNTF mRNA in the various CNS regions paralleled the levels of ciliary neuronal survival activity in these regions (Fig. 1 b) with the exception of the cerebellum, which contained lower levels of ciliary survival activity as those expected from CNTF–mRNA levels.

CNTF Immunoreactivity Is Confined to a Subpopulation of Astrocytes in Primary Rat Brain Cultures

In previous experiments it had been demonstrated that rat brain, type 1–like astrocytes in culture produce CNTF-like molecules which induce O-2A progenitor cells to express GFAP and promote the survival of chick ciliary neurons (Lillien et al., 1988). That this biological activity is indeed

CNTF is supported by the observation that primary cultures of rat brain astrocytes express CNTF–mRNA (Stöckli et al., 1989). However, these investigations did not indicate whether all type 1–like astrocytes in culture, or only a subpopulation, produce CNTF. To address this question, we used a rabbit antiserum produced against a synthetic peptide (peptide I), selected from AA 127-153 of the rat CNTF amino acid sequence. This antiserum (Antiserum 1) and an antiserum against the COOH-terminal part of the CNTF molecule (AA 186-199 of the rat CNTF amino acid sequence, Antiserum 2) recognized a single band in Western blots of extracts of rat sciatic nerve or cultured brain cells (Fig. 2 a). In primary cultures of rat brain, which consisted predominantly of GFAP+ cells, only a relatively small subpopulation of GFAP+ cells were strongly stained by Antiserum 1 (Fig. 3). The CNTF+ cells had the morphological appearance of type 1–like astrocytes. The majority of the GFAP+ cells were not stained at all, or exhibited only very weak staining. Similarly, GFAP⁻ fibroblast-like cells, as well as cells with the morphology of oligodendrocytes or neurons, were not labeled by the antiserum.

Developmental Expression of CNTF mRNA in the Rat Optic Nerve

It was found previously that a CNTF-like, GFAP-inducing activity is present in rat optic nerve extracts starting on postnatal day 8 (PND8) and reaches maximal levels by postnatal weeks 3–4 (Hughes et al., 1988). As shown in Fig. 4 a, this increase in CNTF-like biological activity is preceded by a corresponding increase in CNTF mRNA levels. In Northern blots of rat optic nerve an unambiguous positive signal for CNTF mRNA did not become apparent before PND4. At PND5 a faint signal was detectable and then a very rapid increase in CNTF mRNA occurred by PND7. Maximal levels (corresponding to those in the adult optic nerve) were reached by PND10. The apparent drop in CNTF mRNA levels observed after PND10 is probably because of dilution by the rapid increase in the number of differentiated, myelin forming oligodendrocytes which takes place during the second postnatal week, rather than to a real decrease in CNTF synthesis by the cells producing this factor within the optic nerve.

Localization of CNTF immunoreactivity in sections of adult rat sciatic nerve, optic nerve, and olfactory bulb was done using CNTF antiserum 1 (Anti Peptide I) or a mAb against CNTF (see Fig. 6). Schwann cells were specifically stained in the sciatic nerve (Fig. 5, a–d). In the optic nerve (which contains 5–10 times lower levels of CNTF than the sciatic nerve), cells with astrocyte-like morphology were CNTF+ (Fig. 5, e–h). Unfortunately, it was not possible to costain for GFAP, because the unspecific background became too high. In the olfactory bulb cells forming the glial limiting membrane and cells within the glomerular cell layer were CNTF+ (Fig. 6, b–d). Only few cellular structures within the external plexiform layer were labeled with the anti-CNTF mAb. In contrast, GFAP+ cells are present in all layers of the olfactory bulb (Fig. 6 f). Since double staining with anti-GFAP and anti-CNTF antibodies was not technically possible in tissue sections, we dissociated a 3-wk-old rat olfactory bulb and stained the cells against CNTF and GFAP after a 2-h culture period (Fig. 6, g–i). CNTF staining
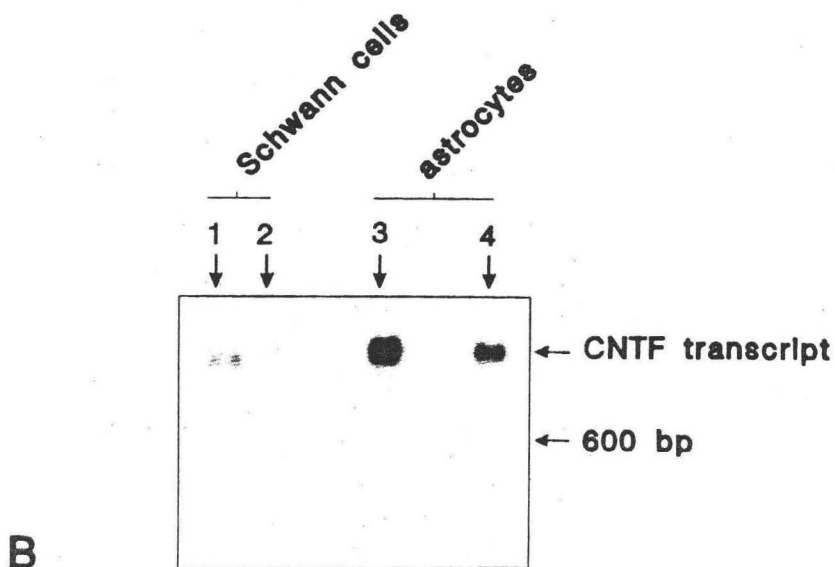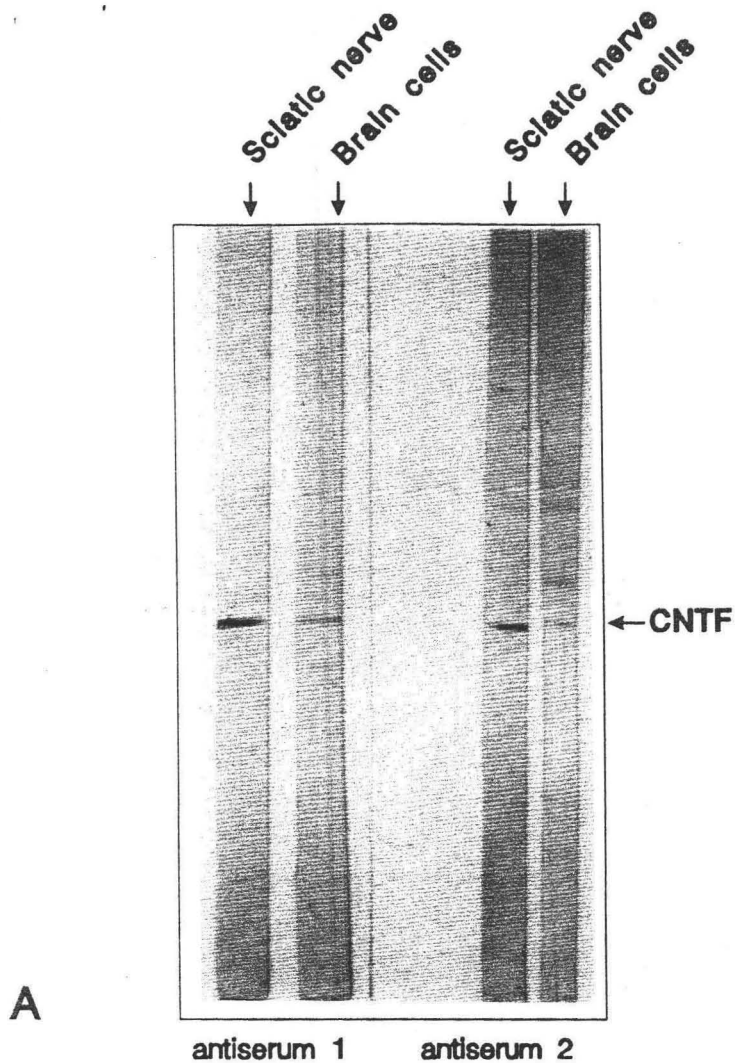
A

antiserum 1   antiserum 2

B

Figure 2. Detection of (*A*) CNTF-immunore-activity and (*B*) CNTF mRNA in cells of the CNS and PNS. (*A*) Extracts of adult rat sciatic nerve (*sciatic nerve*) and new-born rat brain cell cultures (*brain cells*) were blotted onto nitrocellulose. After incubation with antisera (*1*) against CNTF-peptide I; and (*2*) CNTF-peptide II, one major band of about 22-kD size is detected in both extracts. (*B*) Northern blot analysis of cultured Schwann cells (Matsuoka et al., 1991) or astrocytes (Spranger et al., 1990). Total RNA was extracted from cultured new born rat sciatic nerve Schwann cells. 20 μg (*1*) or 10 μg (*2*) were loaded per lane. Comparable quantities of total RNA from 30-d-old cultures of new born rat brain astrocytes are shown in *3* and *4*.
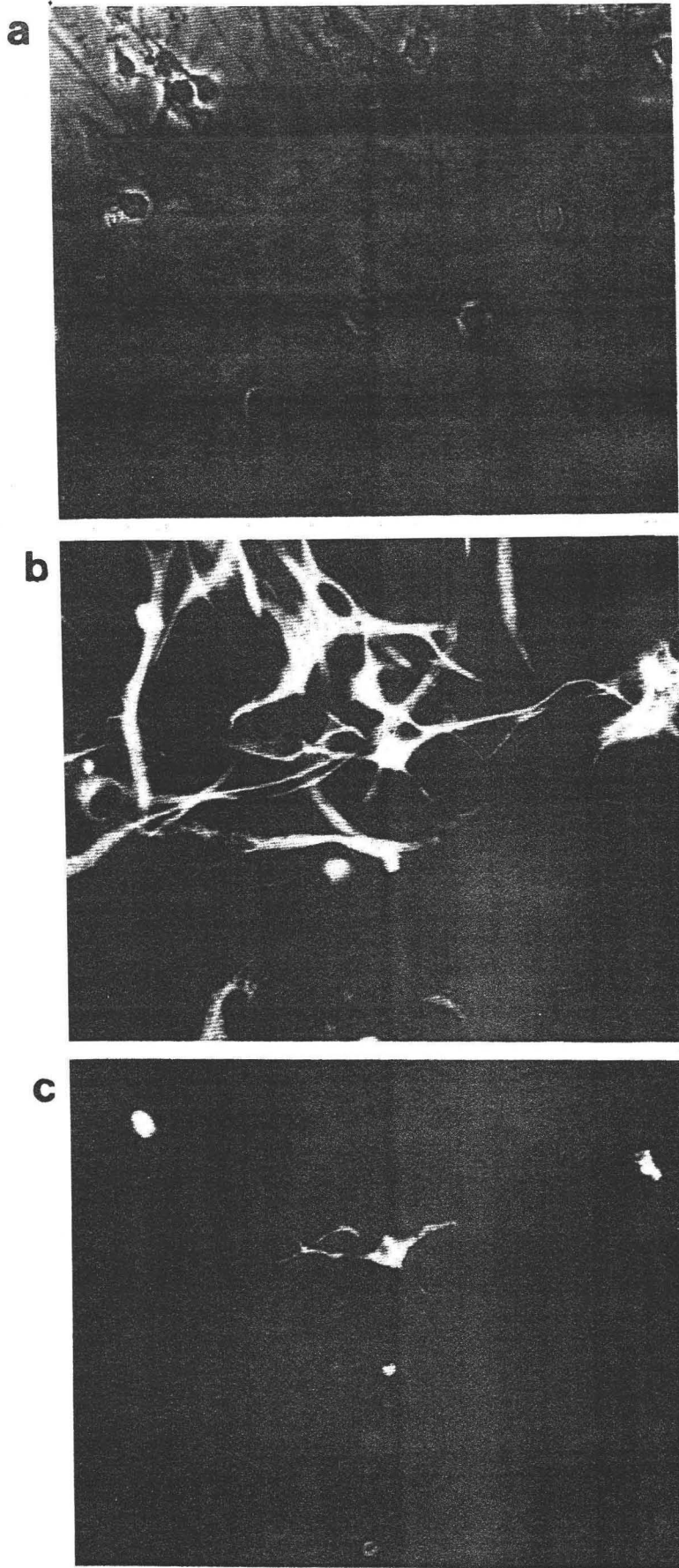
Figure 3. Immunolocalization of CNTF in brain cell cultures. 2-wk-old cultures of new born rat brain cells were fixed and double stained for GFAP and CNTF. (*a*) Phase contrast; (*b*) GFAP immunoreactivity; and (*c*) CNTF immunoreactivity. Bar, 25 μm.
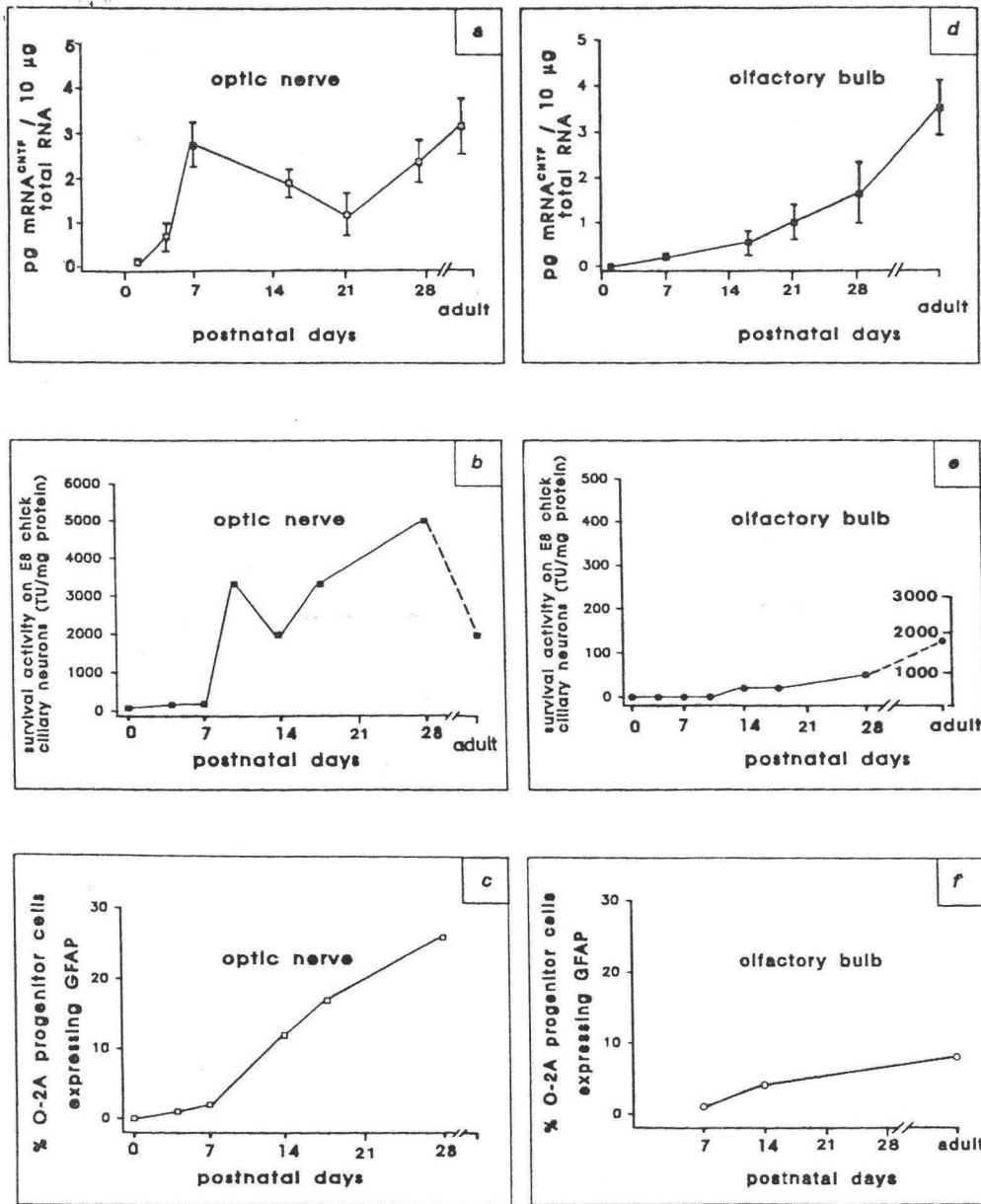
Figure 4. Time course of CNTF expression in the optic nerve and the olfactory bulb during postnatal development of the rat. (*a* and *d*) CNTF mRNA levels are shown as absolute mRNA quantities ± SD found per 10 μg of total RNA of the respective tissue. (*b* and *e*) Ciliary neuron survival activity from rat optic nerve and olfactory bulb extracts. The experimental methods were the same as described in the legend for Fig. 1 *b*. Since the levels of CNTF-like activity increased more than 10-fold between day 28 and adult animals (200 g), an additional scale on the right side of *e* was inserted. (*c* and *f*) Induction of GFAP in cultured 0-2A progenitor cells. The values shown indicate the percentage of GFAP positive progenitor cells after 24 h in culture with 1 μg/ml protein from optic nerve (*c*) and olfactory bulb (*f*) extracts prepared from postnatal rats of various ages.

is detectable only in subpopulations of GFAP positive cells, indicating that a subpopulation of astrocytes produces CNTF within the olfactory bulb.

Developmental Time Course of CNTF mRNA Expression in the Olfactory Bulb Is Different from that in the Optic Nerve

To evaluate whether CNTF expression during CNS development is regulated in a common manner in the different regions of the brain, the developmental expression of CNTF mRNA was also investigated in the olfactory bulb. On PND7, where the maximal level of CNTF mRNA is already reached in the optic nerve, only a faint signal was detectable in the olfactory bulb; the levels increased continuously reaching maximal levels in adult rats (Fig. 4 *d*). The difference in the developmental time course of CNTF–mRNA between the olfactory bulb and the optic nerve became even more apparent

when ciliary neuron survival or GFAP-inducing activity was determined (Fig. 4, *b*, *c*, *e*, and *f*). In extracts of olfactory bulbs CNTF-like activity was extremely low up to postnatal day 28 and significant levels of ciliary survival activity or GFAP-inducing activity could only be detected in extracts of adult rat olfactory bulbs.

PCR Analysis of CNTF mRNA in Early Stages of Rat Embryonic Development

To determine whether there was an additional peak of CNTF expression in earlier developmental stages, total RNA was extracted from whole embryonic day 9 (E9) rat embryos and from the brain and hind limbs of E18 embryos. Northern blot analysis (with a detection limit of 130 femtograms) revealed no specific signal for CNTF mRNA in these tissues (Fig. 7 *a*). Additional studies were performed using PCR techniques, which are at least 10 times more sensitive. The RNA

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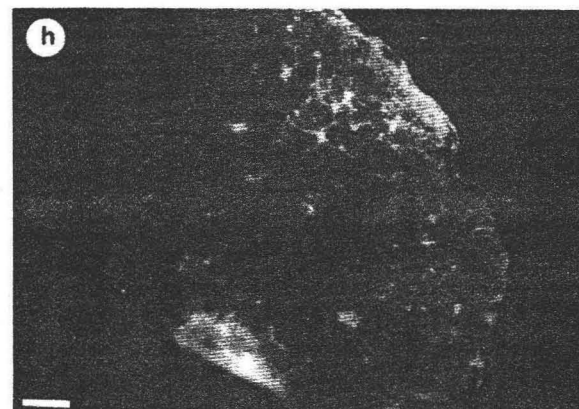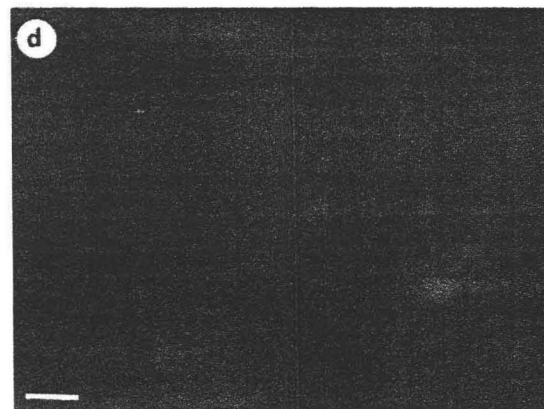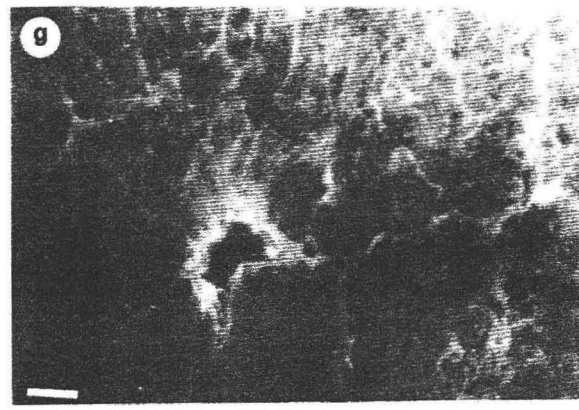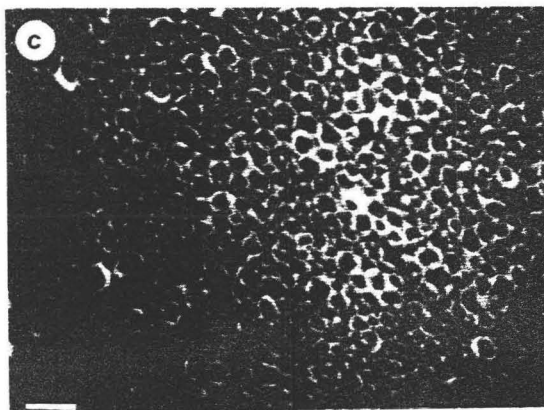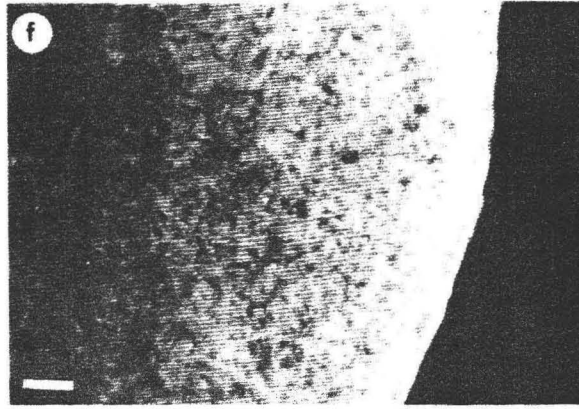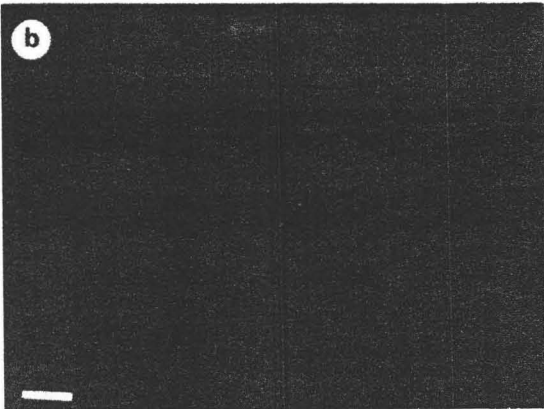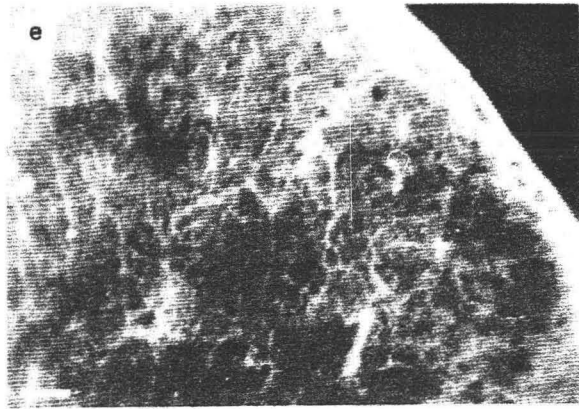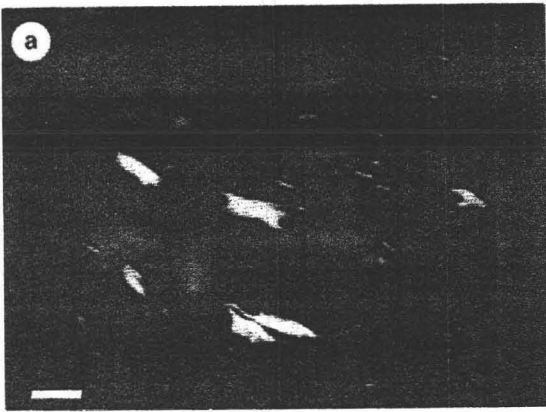
from the embryonic tissues was used for reverse transcription and subsequent PCR reactions; after 17 cycles the reaction products were analyzed by gel electrophoresis. No band was detectable at the expected size of 600 bp (Fig. 7 b).

CNTF-like Activity in Extracts of Adult Rat Optic Nerve can be Precipitated by an Antiserum Produced against CNTF-derived Synthetic Polypeptide

Prot-A-Sepharose was incubated with an antiserum directed against a 14 amino-acid synthetic peptide (peptide II) corresponding to the COOH-terminus of CNTF (amino acid 186-199). After washing, the antibody-loaded Prot–A Sepharose was incubated with various dilutions of an optic nerve extract prepared from 3–4-wk-old rats. After centrifugation, the supernatants were tested for their ability to induce GFAP immunoreactivity in O-2A progenitors in cultures of newborn optic nerve cells. As shown in Fig. 8, more than 95% of the GFAP-inducing activity was removed from the optic nerve extracts, whereas Prot-A-Sepharose loaded with preimmune serum removed very little of the activity.

Discussion

CNTF exhibits a broad spectrum of biological actions on neural cells in culture: it promotes the survival of a variety of embryonic neurons and influences the differentiation of both developing neurons and glial cells (Manthorpe and Varon, 1985; Ernsberger et al., 1989; Saadat et al., 1989; Hughes et al., 1988; Lillien et al., 1988). However, the function(s) of CNTF in normal development is uncertain, especially since it appears to be a cytosolic rather than a secretory molecule (Lin et al., 1989; Stöckli et al., 1989). To act as an extracellular signal in vivo, CNTF would presumably have to be released from degenerating cells, or from healthy cells by an as yet unidentified, nonconventional release mechanism, as has been demonstrated to be the case for interleukin-1 and plasminogen activator inhibitor 2 (Kostura et al., 1989; Rubartelli et al., 1990; Belin et al., 1989). In evaluating the possible role of CNTF in neurodevelopment, it is important to determine which neural cells make the protein and when they begin to produce it. In the present investigation, we have provided evidence that CNTF mRNA and protein are synthesized primarily by glial cells–Schwann cells in the PNS and astrocytes in the CNS.

In earlier investigations (Manthorpe and Varon, 1985), survival activity for ciliary neurons was detected in many embryonic and adult tissues of mammals and chicks, including rat skeletal muscle (Hill et al., 1983), rat brain (Nieto-Sampedro et al., 1982), rat peripheral nerve (Ebendal et al., 1977), chick heart (Adler et al., 1979; Ebendal, et al., 1979), and other tissues. However, the purification of ciliary neurotrophic activity demonstrated that there are at least three different proteins which have similar neuronal survival-promoting activities on chick ciliary neurons: (a) acidic
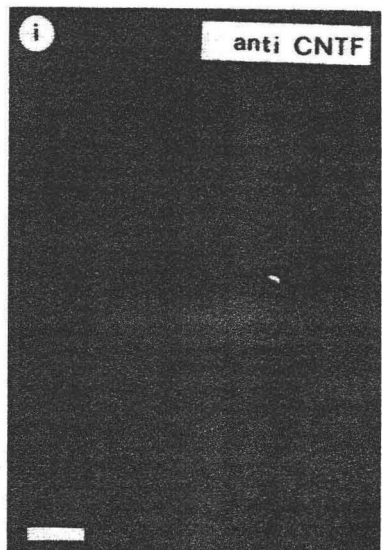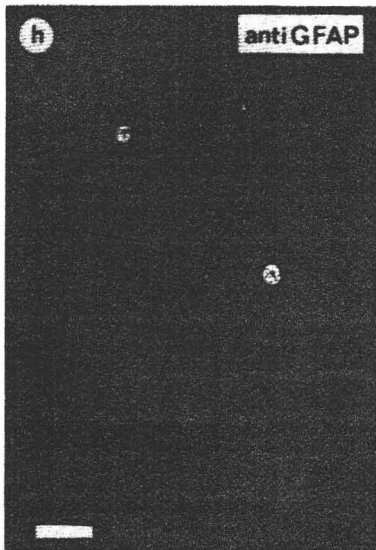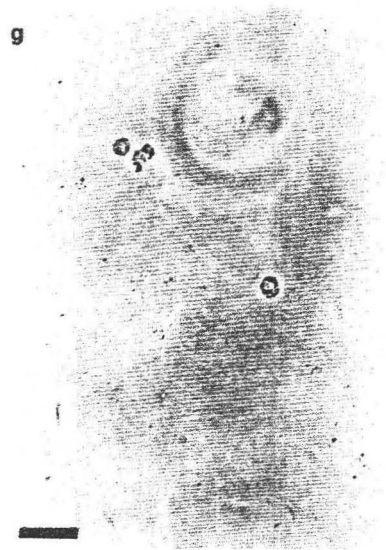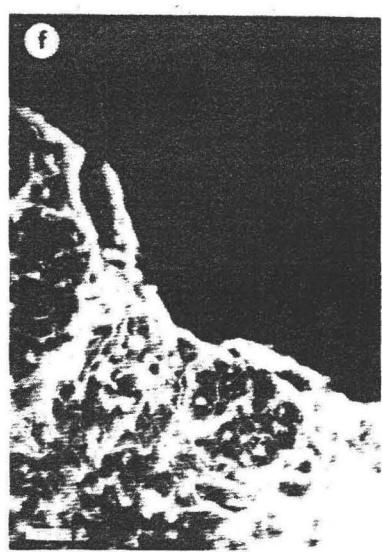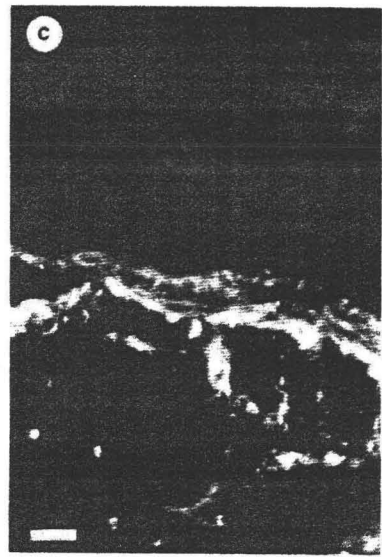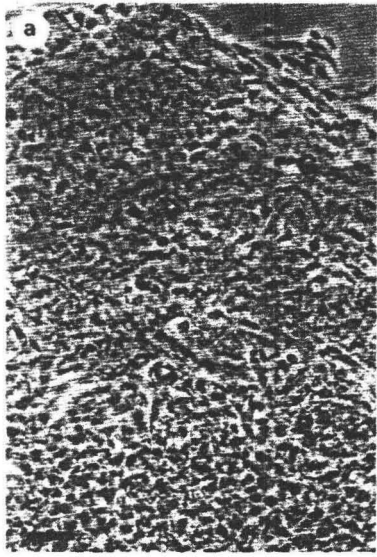
FGF, which was shown to be responsible for the survival activity isolated from bovine heart tissue (Watters and Hendry, 1987); (b) basic FGF, (Unsicker et al., 1987), which was shown to be present in substantial quantities in extracts of skeletal muscle (McManaman et al., 1989), in the CNS, and in cultures of astrocytes (Hatten et al., 1988); and (c) CNTF. The expression of CNTF mRNA is much more restricted than FGF mRNA, but as CNTF mRNA and protein are both present in substantial quantities in Schwann cells of peripheral rat nerves, the ciliary survival activity found in skeletal muscle, skin, and heart could well result, at least partially, from CNTF in the Schwann cells of sensory, autonomic, or motoneurons innervating these tissues. Thus, the CNTF-like biological activity found in many tissues of adult rats could result from other molecules such as acidic FGF and basic FGF, or from CNTF localized in innervating nerve fibers. In any case, it is clear that the identification of CNTF in tissue samples cannot rely simply on the determination of survival activity for ciliary neurons, but has to be complemented by immunological studies and/or Northern blot analysis.

The present experiments have shown that CNTF mRNA and protein first appear postnatally in the rat (Stöckli et al., 1989). We could not find evidence for the expression of CNTF mRNA in tissues of E10 and E18 rat embryos after PCR amplification, supporting the concept that the physiological function of CNTF is restricted to the postnatal period in the rat.

CNTF has been shown to have a powerful survival activity on purified spinal motoneurons from embryonic chick (Arakawa et al., 1990). However, the time period of physiological motoneuron cell death in rats is over by the time CNTF starts to be expressed both in the periphery and in the CNS. On the other hand, the time course of CNTF expression in rat peripheral nerves is compatible with its function as a Schwann cell–derived "lesion factor" that keeps motoneurons alive until they can grow back to their muscle targets. This would explain why motoneurons survive axotomy once CNTF is expressed, but fail to do so before CNTF is expressed (Sendtner et al., 1990).

Reports on the action of CNTF on other populations of CNS neurons are controversial. It has been reported, for example, that CNTF promotes the survival of chick retinal ganglion cells (Lehwalder et al., 1989), and in mixed cultures of E8 chick retina cells, that it increases the level of ChAT, probably in amacrine cells (Hofmann, 1988a,b). CNTF, however, does not promote the survival of embryonic or newborn rat retinal ganglion cells (J. E. Johnson, personal communication), under experimental conditions where BDNF does so (Johnson et al., 1986). In the present study we could not detect CNTF mRNA by Northern blot analysis in the retina, and retinal extracts had very little survival activity for ciliary neurons (Fig. 1), making it unlikely that retinal-derived CNTF plays a role in normal retinal development, at least in the rat.

Figure 5. Immunolocalization of CNTF in sections of adult rat sciatic and optic nerve. In a and b longitudinal sections, in c and d transverse sections of adult rat sciatic nerve are shown. (e–h) Transverse sections of 24-d-old rat optic nerve. Controls with preadsorption of the antiserum to excess CNTF peptide are shown in b, d, and f. Within the sciatic nerve, CNTF immunoreactivity is located within the cytoplasm of Schwann cells. CNTF-immunoreactive cells within the optic nerve are approaching the glial limiting membrane (e) and blood vessels within the nerve (g). Bars: (a–d) 25 μm; (e–g) 15 μm; (h) 100 μm.

a

CNTF transcript →

recovery standard →
(600 bp)

1 2 3 4 5 6
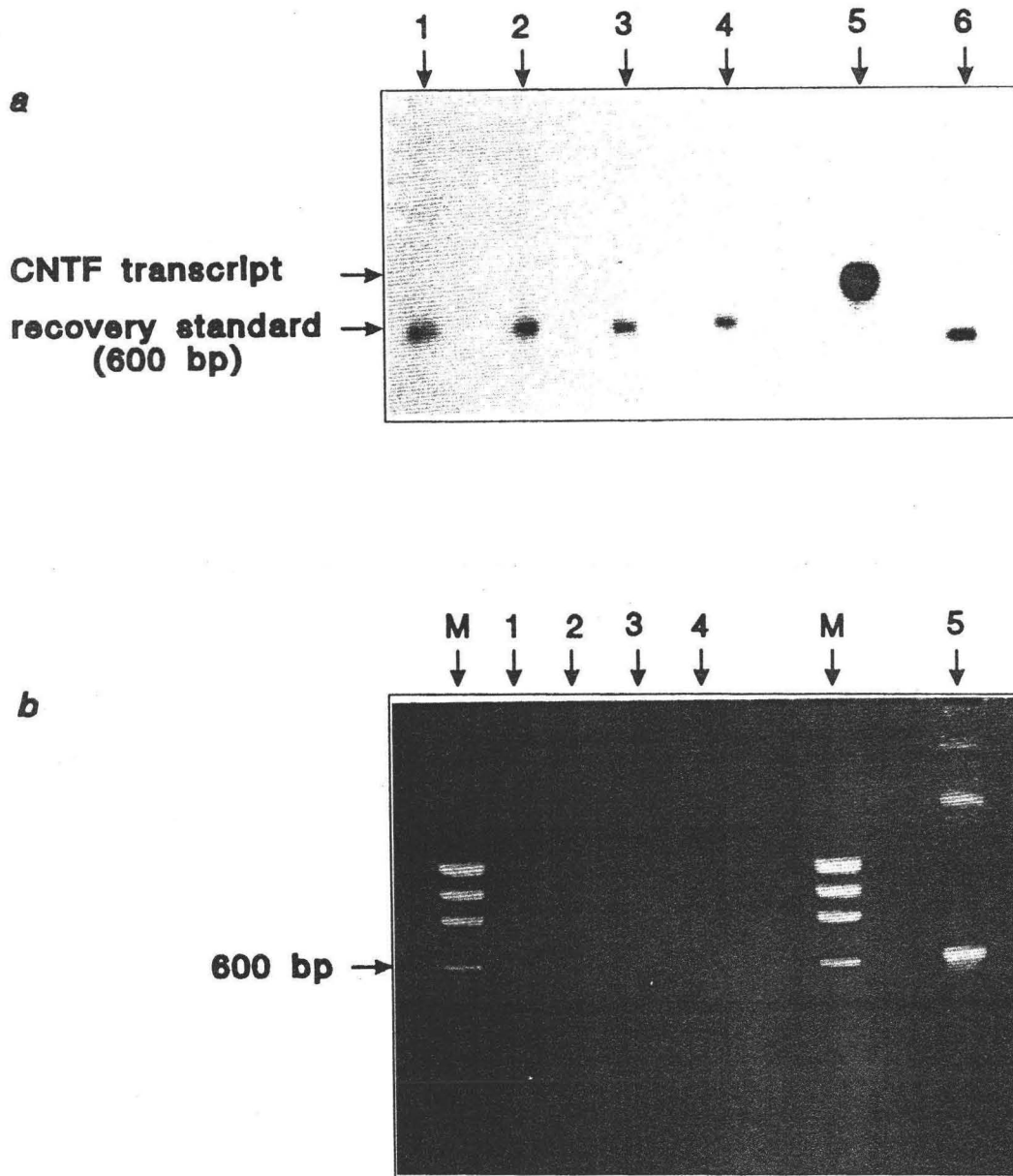

b

M 1 2 3 4 M 5

600 bp →

Figure 7. Analysis of CNTF expression during prenatal development of the rat. (*a*) Northern blot analysis of embryonic tissue. (Lane *1*) E18 brain; (lane *2*) E18 hind limb; (lane *3*) E9 head; (lane *4*) E9 trunk; (lane *5*) adult sciatic nerve (control); (lane *6*) 6.5 pg of an in vitro transcribed CNTF-RNA recovery standard (600 bp). (*b*) Gel electrophoresis after 17 cycles of the polymerase chain reaction (PCR). The embryonic tissues used for the reverse transcription and subsequent PCR are indicated. (lane *1*) E18 brain; (lane *2*) E18 hind limb; (lane *3*) E9 head; (lane *4*) E9 trunk; (lane *5*) adult rat sciatic nerve (control); (*M*) molecular weight marker ($\varphi \times 174$ HaeIII DNA).

The highest levels of CNTF mRNA in the rat CNS are found in the optic nerve and olfactory bulb (Fig. 1). As the optic nerve does not contain neuronal cell bodies, CNTF is likely to be made by glial cells in the nerve. Indeed, in sec-tions of optic nerve of 24-d-old rats, CNTF immunoreac-tivity (Fig. 5, *e–h*) was detected in cells which morphologi-cally resembled type 1 astrocytes (Miller et al., 1985). Although the direct identification of these cells by double im-

Figure 6. Immunolocalization of CNTF in sections and cultured cells of adult rat olfactory bulb. (*a*) Section of the olfactory bulb showing the vomeronasal nerve fiber layer (*F*), the glomerular layer (*G*), and the external plexiform layer (*E*). (*b*) Immunofluorescence using the anti-CNTF mAb 4-68 identifies CNTF immunoreactivity within cells of the glomerular layer of the same section. (*c*) These cells are con-tributing to the glial limiting membrane, others are located periglomerularly. (*d*) At higher magnification processes of the CNTF+ cells are detectable. These processes are also located in the periphery and do not project into the glomeruli. (*e*) Control section of the same area. The CNTF antibody was preadsorbed to recombinant CNTF. (*f*) Anti-GFAP immunostaining of the same area. (*g*) Phase contrast picture of three cells of short-term cultures (2 h) of 3-wk-old rat olfactory bulb. (*h*) Two of these cells are GFAP+. (*i*) One of these GFAP+ cells is also labeled against CNTF. Bars: (*a–c, g–i*) 25 µm; (*d–f*) 15 µm.
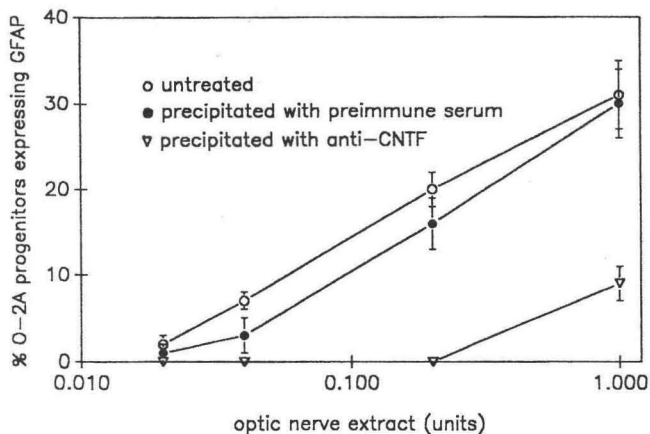
Figure 8. Immunoprecipitation of optic nerve extract with an antiserum against a COOH-terminal peptide of CNTF. Adult rat optic nerve extract was incubated with an anti-CNTF peptide II antiserum ($\triangledown$) or a preimmune serum ($\bullet$), both being adsorbed to Prot-A-Sepharose. After centrifugation the supernatants were tested for their ability to induce GFAP in O-2A progenitor cells in cultures of newborn rat optic nerve cells. A control with untreated optic nerve extract is shown in curve ($\circ$). One unit is defined here as the amount of untreated optic nerve extract necessary for the induction of a maximal number of GFAP positive cells in cultured O-2A precursor cells.

munolabeling was technically not possible (see results), we have shown that a subpopulation of GFAP positive type 1–like astrocytes in cultures of new-born rat brain expressed CNTF immunoreactivity (Fig. 3). Similarly, in short-term cell cultures of three week old rat olfactory bulb, only GFAP positive cells were colabeled against CNTF (Fig. 6, *g–i*). We could not detect CNTF immunoreactivity in oligodendrocytes, fibroblasts, or neurons in these cultures. These observations lend strong support to the hypothesis that astrocytes are the main producers of CNTF in the optic nerve, the olfactory bulb, and probably elsewhere in the rat CNS (Lillien and Raff, 1990).

The time course of expression of CNTF in the optic nerve is of special interest in that it has been proposed that the onset of CNTF production initiates type 2 astrocyte development (Hughes et al., 1988; Lillien et al., 1988). The time of appearance of type 2 astrocytes in cultures of embryonic brain cells coincides with the appearance of a CNTF-like, GFAP-inducing activity in these cultures (Lillien et al., 1988). Moreover, the time of appearance of cells with the antigenic properties of type 2 astrocytes in cell suspensions of developing optic nerve also coincides with the appearance of such CNTF-like activity in the nerve. In the present study, we have shown that both CNTF mRNA and CNTF-like bioactivity are expressed in the developing optic nerve with the same time course (Fig. 4, *a–c*). Moreover, we found that more than 95% of the GFAP-inducing activity in optic nerve extracts can be precipitated by an antiserum directed against a CNTF COOH-terminal peptide (Fig. 8), providing the strongest evidence so far that this activity is due to CNTF. It has been shown previously that CNTF does not act alone to induce O-2A progenitor cells to differentiate into type 2 astrocytes in culture: it synergizes with signaling molecules that are bound to the extracellular matrix (Lillien et al., 1990). Whereas all of the signals required to induce type 2

astrocyte differentiation in vitro are present in the optic nerve (Lillien and Raff, 1990), it remains to be demonstrated that type 2 astrocytes actually develop in vivo.

The developmental expression of CNTF in the olfactory bulb starts later than in the optic nerve (Fig. 4, *d–f*), indicating that not only the extent of the expression, but also the developmental time course differs from one CNS region to another. The cellular source within the olfactory bulb is a subpopulation of GFAP+ cells, some of which contribute to the glial limiting membrane while others are located in close vicinity to the glomeruli. Similar cells within the superficial layer of the olfactory bulb have been described by Raisman (1985). Evidence from lesion experiments (Doucette et al., 1983) suggests that such cells are important for the regrowth of lesioned olfactory nerve back into the olfactory bulb to form glomeruli. These specialized astrocytes ensheath the vomeronasal axons along their course within the CNS to the glomeruli. It is an intriguing possibility that the CNTF synthesized by these cells could assist the regeneration of ingrowing olfactory nerves, either physiologically and/or after lesion.

In summary, we have shown that CNTF is produced by a subpopulation of astrocytes within the olfactory bulb, the optic nerve, and possibly in other regions of the brain during postnatal development. Although the absence of a hydrophobic leader sequence and its nonrelease from transfected eukaryotic cells suggest that CNTF is a cytosolic molecule, its distinct regional and developmental expression pattern indicates that it could play an important role in the developing and/or regenerating CNS, especially in the optic nerve and olfactory bulb.

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