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Neurons Express Ciliary Neurotrophic Factor mRNA in the Early Postnatal and Adult Rat Brain

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The regional and subcellular localization in the central nervous system (CNS) of postnatal day 5, day 15, and adult rats of ciliary neurotrophic factor (CNTF) mRNA was examined by in situ hybridization with biotinylated riboprobes. Probe specificity was determined by Northern blot analysis of poly(A)+ RNA extracted from adult rat brain using digoxigenin labeled riboprobes and chemiluminescent detection. Both a 4 kb and a 1.2 kb transcript were detected in the cortex and brainstem. In situ hybridization revealed that CNTF mRNA was widely distributed in neurons and glia throughout the CNS at each of the developmental time points. The density of the neuronal hybridization signal was found to be greater in neuronal nuclei than in their cytoplasm. In the nucleus of most neurons, CNTF mRNA distribution was concentrated in a perinucleolar fashion. Alternate sections from the same animals, which were incubated with a specific polyclonal antibody against a CNTF peptide fragment, revealed that both neurons and glia in postnatal day 5, day 15, and adult rat brain were immunoreactive for CNTF.

Key words: ciliary neurotrophic factor, in situ hybridization, biotinylated riboprobes, postnatal development, chemiluminescent detection

INTRODUCTION

Previous studies have reported ciliary neurotrophic factor (CNTF) mRNA expression in myelinating Schwann cells in intact sciatic nerves (Dobrea et al., 1992; Friedman et al., 1992; Sendtner et al., 1992; Seniuk et al., 1992) using Northern blot, in situ hybridization, or RNAse protection analysis. In the central nervous system (CNS), a widespread distribution for CNTF mRNA has been reported throughout the adult rat brain, with highest levels found in the optic nerve and olfactory bulb (Stockli et al., 1991; Rudge et al., 1992) as determined by densitometric analysis of Northern blots. Northern blot analysis has also been used to detect CNTF mRNA in cultures of primary "type 1" astrocytes and optic nerve tissue (Lillien and Raff, 1990; Stockli et al., 1991). In situ hybridization has localized CNTF mRNA to process-bearing astrocytes in heterogeneous glial cultures (Seniuk et al., 1994) and to "reactive" astrocytes in vivo (Ip et al., 1993). Since CNTF mRNA is readily detectable in total RNA extracts from primary glial cell cultures originating from the optic nerve, cortex, hippocampus, etc. (Lillien and Raff, 1990; Rudge et al., 1992; Carroll et al., 1993); it has been generally assumed that CNTF mRNA detected in the CNS is expressed only in non-neuronal cells (Stockli et al., 1991; Manthorpe et al., 1993). However, Rudge and coworkers (1992) were able to detect CNTF mRNA in neurons cultured from rat hippocampal tissue.

Using an affinity purified polyclonal antibody to a peptide fragment of CNTF (amino acids 131–147) we have recently shown that CNTF immunoreactivity is localized to both neurons and glia (Henderson et al., 1994) in the adult rodent CNS. It is therefore important to determine whether, in fact, neurons in the CNS are capable of synthesizing CNTF or whether neuronal CNTF can only be derived from nearby glia. In order to resolve this issue, we have used biotin labeled riboprobes with colorimetric detection. Non-isotopically labeled probes provide a level of resolution not attainable with ^{35}S labeled probes, particularly in areas of high cell density where it would be impossible to distinguish neuron-specific message from glial-specific message due to grain scatter which accompanies radioactive in situ hybridization. We now report that CNTF mRNA is detectable in rat brain neurons by this method and that the distribution of CNTF mRNA appears to correspond to that of CNTF immunolocalization for adult rat brain, as well as for postnatal days (PD) 5 and 15.

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MATERIALS AND METHODS

Preparation of Non-Isotopically Labeled Riboprobes

A 0.6 kb rat CNTF cDNA inserted at the Bam HI site in pGEM7zf- (Seniuk et al., 1992) was used for probe synthesis. Full length antisense probes were transcribed and biotin-CTP labeled from a Hind III-linearized plasmid template using a non-isotopic RNA labeling kit (GIBCO/BRL, Gaithersburg, MD). Plasmid linearized with Nco I provided the template for the sense probe transcription/labeling which was used as a control for all antisense hybridizations. Probes were purified on a Sephadex G50 column, ethanol precipitated, and resuspended in diethylpyrocarbonate (DEPC)-water before use. A small aliquot was run on a 1% agarose gel against a known standard in order to estimate the amount of probe synthesized and to examine the quality of the transcription. Similarly, digoxigenin (DIG)-uridine triphosphate (UTP) labeled antisense riboprobes were also transcribed from Hind III-linearized template using the DIG-RNA labeling kit (Boehringer Mannheim, Indianapolis, IN) and purified on a G50 column and ethanol precipitated before use. Probe concentration was estimated by chromogenic or chemiluminescent detection of a probe dilution series and compared against a DIG labeled standard supplied with the kit.

In Situ Hybridization

PD5, PD15, and adult (4-month-old) rats were perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), the brains were dissected free, postfixed overnight at 4°C, then rinsed through several changes of cold PBS followed by cryoprotection in 30% sucrose in PBS. Brains were frozen in isopentane and stored at −70°C until needed. Frozen, 10 μm thick, serial, coronal sections were thaw-mounted onto APTEX (Sigma, St. Louis, MO) coated slides and stored at −20°C for no longer than 3 weeks before use.

When required, slides were removed from the freezer and washed twice in PBS. Sections from PD5 and PD15 rat brains were digested for 40 sec and 1.5 min, respectively, at 37°C with proteinase K (5 μg/ml in Tris/EDTA buffer, pH 8.0), while sections from adult rat brain were digested for 3 min. Enzyme activity was halted with an ice-cold PBS bath. Slides were incubated for 30 min in 0.1 M glycine/0.1 M Tris (pH 7.0–7.2), then dehydrated through a series of ethanol baths and left to air dry for 10 min. Sections were prehybridized for 30 min at 46°C with hybridization buffer (50% formamide, 4× SSC (20× SSC = 3 M NaCl/0.3 M sodium citrate), 10 mg/ml dextran sulfate, 1 mg/ml yeast tRNA, and 1× Denhardt's (50× Denhardt's = 10 g/l Ficoll, 10 g/l polyvinylpyrrolidone, 10 g/l bovine serum albumin). Excess hybridization solution was blotted away and 50–80 μl of hybridization buffer containing probe, at an estimated concentration of 200–400 ng/ml, was added to each slide. Antisense and sense probes were run simultaneously on alternate sections so that they would be subjected to identical hybridization conditions and stringency washes. Sections were covered with a parafilm "coverslip" and left to hybridize overnight at 46°C. Sections were washed as follows: 1) 2× SSC, twice at room temperature, 5 min each, 2) 2× SSC at 65°C for 30 min, 3) 0.2× SSC twice at room temperature, 5 min each, 4) 0.2× SSC at 42°C for 30 min, 5) 0.1× SSC at room temperature for 10 min. RNAse-free solutions were used up until this point. Following stringency washes, slides were rinsed with PBS and then incubated with avidin-horseradish peroxidase (Elite kit, Vector Labs, Burlingame, CA) for 30 min. Slides were rinsed with PBS and then sense and antisense slides incubated simultaneously with diaminobenzidine (DAB), 1.2 mg/ml mixed v:v 0.02% hydrogen peroxide. The chromogenic detection reaction using DAB, horseradish peroxidase and hydrogen peroxide can be easily standardized and has been shown to behave as a linear function which is driven to saturation after 5 min when a molar excess (equivalent to 1.2 mg/ml) of DAB is used (Reis et al., 1982; Seniuk et al., 1990).

In Situ Hybridization Controls

There is no single control which will establish the specificity of an in situ hybridization (Angerer et al., 1987b; Tecott et al., 1987; Watson et al., 1987), so that several tests should be performed in order to support the pattern of hybridization signal as being specific for the mRNA of interest. We used the following tests of specificity: 1) Confirm probe specificity by Northern blot analysis to identify the transcript of appropriate length in the tissue being examined. 2) Determine that the hybridization signal is RNA specific, but not DNA specific. Although prehybridization digestion with DNAse I can be performed, it does not necessarily produce a complete digest and so is not entirely reliable (Angerer et al., 1987a). As an alternate to DNAse I digestion, single stranded sense RNA probes (identical to the message) were used. Since DNA is generally transcribed asymmetrically, the sense probe remains complementary to the opposing strand and has therefore been considered the best control against non-specific hybridization to DNA (DeLeon et al., 1983). Further, the sense probe is also a full length transcript with identical G-C content (and presumably "stickiness") and identical opportunity for biotin-CTP incorporation. Therefore, the sense probe would be expected to exhibit similar hybridization kinetics to the antisense probe under identical conditions. 3) Establish that the observed hybridization signal is RNA specific and does not reflect a non-specific association

with other cellular components. Prehybridization RNAse A digestion results in cleavage of single stranded RNA at 3′ phosphate linkages leaving terminal pyrimidine 3′ phosphates and oligonucleotides and thereby eliminating any mRNA for specific hybridization. 4) Posthybridization RNAse digestion to help establish that the detected signal reflects only double stranded hybridization complexes which should be "protected" from RNAse cleavage. We and others (Turnbow and Garner, 1993) have found that RNAse A is able to digest biotin labeled riboprobes. Apparently biotin-CTP can sufficiently destabilize the RNA-RNA duplex to cause "breathing." This would allow RNAse A to cleave the hybridization complex at the 3′ phosphate linkage of the biotin-CTP. We therefore performed a posthybridization digest with RNAse T1 which cleaves specifically on the 3′ side of G residues. 5) Hybridization of probe to tissues lacking the specific mRNA to assess the contribution of non-specific background to the detected signal and to rule out false positive signal. 6) Colocalization of the mRNA with the protein it encodes by using a specific antibody to the protein. It must be noted that this test does not hold for all cases, either because the protein is below the threshold of detection, or the protein product is accumulated elsewhere.

Northern Blot Analysis

In order to establish probe specificity, poly(A)+ RNA was extracted from freshly dissected adult rat cortex and brainstem (including di- and mesencephalon) using a messenger RNA isolation kit (Stratagene, La Jolla, CA). Five micrograms each of poly(A)+ RNA from the cortex and brainstem were run on a 1% agarose/0.66 M formaldehyde gel and stained with ethidium bromide to record the position of the 28S and 18S ribosomal RNA bands. The gel was rinsed in 2 changes (15 min each) of 20× SSC and the RNA blotted to a positively charged nylon membrane (Boehringer Mannheim) by capillary transfer with 20× SSC at room temperature for 4 hr. Following transfer, the RNA was ultraviolet (UV)-crosslinked to the membrane and then placed in prehybridization buffer [50% deionized formamide, 5× SSC, 0.1% lauryl-sarcosine, 0.02% sodium dodecyl sulfate (SDS), 2% blocking reagent (Boehringer Mannheim)] for 10 min at 65°C. DIG labeled antisense CNTF riboprobe was boiled for 10 min, iced briefly, then added directly to the membrane in prehybridization buffer at a concentration of approximately 75 ng/ml. Following overnight hybridization at 65°C, the membrane was washed twice for 5 min with an excess volume of 2× SSC/0.5% SDS at room temperature, followed by 2 washes, 15 min each, with an excess volume of 0.1× SSC/0.5% SDS at 65°C. The membrane was then processed for chemiluminescent detection which included incubation with an anti-DIG-alkaline phosphatase antibody (1:10,000) followed by a brief incubation with Lumigen PPD (Boehringer Mannheim). The membrane was then exposed to X-ray film for 3.5 hr at room temperature in order to visualize CNTF mRNA signal. A digitized image of the exposed film was taken using a CCD video camera and Metamorph software (see details below).

Immunocytochemistry

Selected sections which had been hybridized with antisense CNTF probe were then processed for double labeling with monoclonal antibodies to glial fibrillary acidic protein (GFAP) (G3893, Sigma), vimentin (V9 clone, Boehringer Mannheim), or neurofilament protein (SMI 33, Sternberger Monoclonals, Baltimore, MD). Following visualization of the hybridization signal, slides were rinsed with PBS. Sections for GFAP (or vimentin) were treated for 10 min with cold (−20°C) methanol prior to blocking for 20 min with 10% normal horse serum (Vector Labs). Primary antibody incubations were for 1 hr at 37°C diluted with 1% horse serum in PBS: GFAP, 1:800; neurofilament, 1:1,000; vimentin, 1:20. Sections were rinsed, incubated for 30 min with biotinylated horse anti-mouse IgG (3 μg/ml) at room temperature, rinsed, and incubated for 30 min with avidin-alkaline phosphatase (Vector Labs). After a brief rinse, sections were incubated for 25 min in the dark with Vector Red chromogenic agent. The development of the color reaction was halted with distilled water and the slides were dehydrated and permanently mounted. Vector Red allows double labeling on brightfield observation (red reaction product) or with fluorescent excitation (in the range of rhodamine). Sections incubated with polyclonal CNTF antibody were processed as previously described (for details see Henderson et al., 1994).

Image Analysis

Sections were examined on a Polyvar 2 microscope and the images "captured" with Metamorph software (Universal Imaging Software Corp., West Chester, PA) in conjunction with a MATROX frame grabber and CCD video camera (model XC-77). Images were imported into Corel Draw (v. 3.0) to produce the final photoplate. The CNTF hybridization image (see Fig. 6) was obtained by increasing the digital contrast and filtering the normal brightfield image to eliminate the red signal of Vector Red and to allow visualization of the brown DAB component only. The neurofilament signal was observed under UV excitation with a rhodamine sensitive filter which allowed visualization of Vector Red only.

Optical density (OD) measurements for quantitative analysis were also taken from captured images in Metamorph. OD was measured within a box of set size

placed within the cell soma or nucleus of each neuron. Each value was the average of two boxes which were then corrected for background OD contribution. To determine the background, an identical box was placed in the immediate vicinity of each neuron, and the background OD value averaged from two boxes was subtracted from each neuronal cytoplasmic or nuclear value (for details see Seniuk et al., 1994). Non-parametric statistics (Kolmogorov-Smirnov) were performed using Statistica software (Statsoft, Tulsa, OK).

RESULTS

Antisense and sense probe hybridizations were performed simultaneously and timed exactly for PD5, PD15, and adult rat brain sections. High stringency wash conditions were initially established for the adult rat brain which would be taken as the baseline of CNTF mRNA expression. Therefore, all hybridization conditions were identical for all age groups, but optimal for the adult brain. The only difference between groups was a shorter digestion period in proteinase K for the early postnatal tissue, which otherwise could not withstand the entire hybridization protocol if adult tissue digestion conditions were applied.

Several controls were performed to support the specificity of the hybridization signal. In order to establish probe specificity for CNTF mRNA, poly(A)+ RNA was extracted from adult rat brain cortex and brainstem (including di- and mesencephalon) and analyzed by Northern blot analysis (Fig. 1). Antisense riboprobes were labeled with DIG-UTP and the hybridization signal was visualized by chemiluminescent detection according to the manufacturer's suggested protocol. Five micrograms (μg) each of RNA from the cerebral cortex (lane 1) and the brainstem (lane 2) were run on a formaldehyde gel. A larger transcript, approximately 4 kb in size, was readily detectable in both samples. A smaller transcript, approximately 1.2 kb in size, was just detectable in both samples. These transcript sizes correspond to previous reports in the literature for CNTF mRNA (Lin et al., 1989; Stockli et al., 1989; Rudge et al., 1992). There was no apparent difference in signal strength between the cortex and brainstem for either transcript.

CNTF antisense probe hybridizations are shown in facial motoneurons from PD5 (Fig. 2A), PD15 (Fig. 2B), and adult rat (Fig. 2C) brain. Note that the CNTF antisense hybridization signal was clearly distinguishable from background and that there appeared to be more CNTF mRNA in the nucleus of all neurons when compared with the cytoplasmic signal for each postnatal developmental stage. This same pattern was also detectable in other smaller cells in the section which were identified as astroglia by GFAP immunoreactivity on alternate sections (data not shown). In many neurons, nuclear CNTF mRNA appeared to be perinucleolar in its disposition. The clumping effect seen in the cytoplasm of adult motoneurons could reflect subcellular compartmentalization of the cytoplasmic CNTF mRNA or it could be an artifact, due to the proteinase K digestion. This pattern was not observed in all neurons.

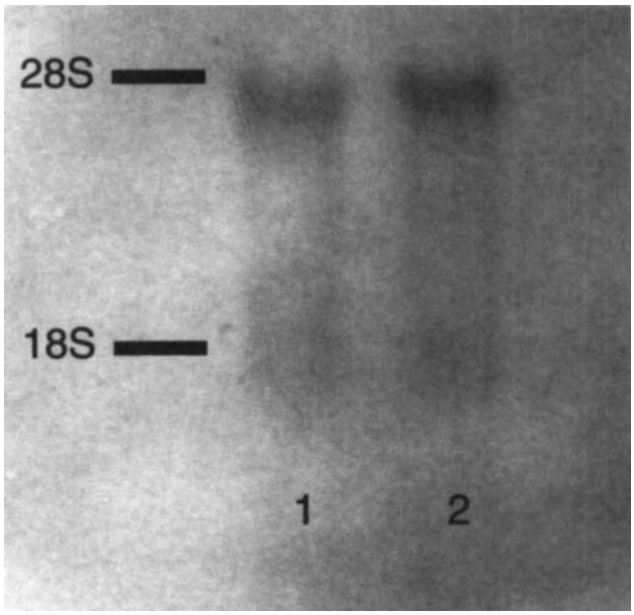


Fig. 1. Chemiluminescent detection of CNTF mRNA in adult cortical and brainstem poly(A)+ extracts with DIG labeled riboprobes. Antisense CNTF riboprobe was hybridized at 65°C to 5 μg each poly(A)+ RNA from adult rat brain cortex **(lane 1)** and brainstem **(lane 2).** Note that both a large 4 kb transcript and a smaller, less dense 1.2 kb transcript are detectable in the adult rat brain. Also note that the signal strength is comparable between the cortex and brainstem for both transcripts.

RNAse digested controls are displayed in Figure 3A–C. One set of slides from each age group was pretreated with RNAse A (20 μg/ml) for 20 min at 37°C to digest all RNA from the tissue section prior to hybridization with the antisense probe. No hybridization signal was detected for any age group examined, indicating that the observed antisense hybridization signal was RNA specific. Figure 3A illustrates a high power, interference contrast micrograph of a section taken through the cerebellum of the adult rat.

Posthybridization digestion with RNAse A has been routinely used with radiolabeled probes to decrease background by digesting all single stranded RNA in the tissue section and to indicate whether detected signal is RNA-RNA specific. In our hands, posthybridization digestion of biotinylated riboprobe-mRNA duplexes were found to be vulnerable to RNAse A digestion (see Materials and Methods). Figure 3B illustrates a section

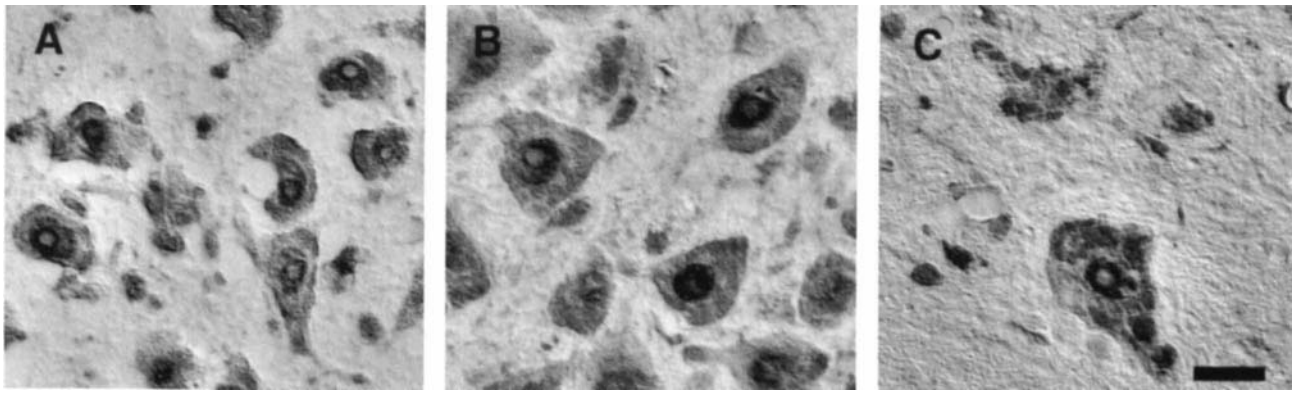


Fig. 2. CNTF antisense probe hybridizations of facial motoneurons in cryosections from PD5 **(A)**, PD15 **(B)**, and adult rat **(C)** brain. CNTF mRNA was found in both the nucleus and cytoplasm of motoneurons for each age group examined. Interference contrast optics were used to provide definition and visualization of cell boundaries. The small cell bodies observed in the section were identified as glial cells by GFAP immunocytochemistry on alternate sections (data not shown). In general, CNTF mRNA was found in greater density in the nucleus, often with a distinct perinucleolar distribution. Bar = 20 μm.

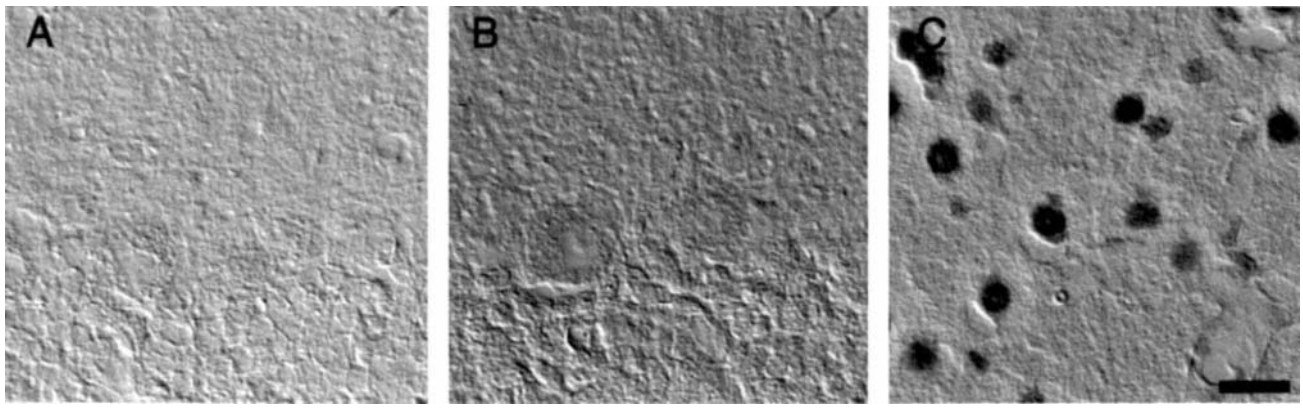


Fig. 3. RNase digests of adult rat brain sections demonstrate RNA specificity of hybridization signal. **A:** Cryosections from the adult rat brain were subjected to RNAse A digest (20 μg/ml, 20 min) prior to hybridization to determine that the hybridization signal was RNA specific. No signal was detectable following antisense probe hybridization. **B:** Sections subjected to RNAse A digest following antisense hybridization resulted in no detectable signal due to digestion of biotinylated probe/mRNA complexes. **C:** Sections subjected to RNAse T1 digest (12.5 U/ml, 30 min) retained specific hybridization signal demonstrating subcellular localization identical to that observed when no RNAse digest was performed. A and B were taken from the adult cerebellum at the level of the facial nucleus; C was taken from the adult cerebral cortex at the level of the medial habenula. Bar = 20 μm.

through the cerebellum of an adult rat demonstrating the complete loss of antisense signal with a 10 min posthybridization incubation with RNAse A (20 μg/ml). Therefore, posthybridization digests with RNAse T1 were performed. Using concentrations of 1–25 U/ml of RNAse T1 for 30 min at 37°C, we found that the hybridization signal was identical to antisense hybridizations without RNAse T1 treatment. RNAse T1 digests were also performed with sense probe hybridizations; again, there was no detectable signal. Figure 3C illustrates a section through the cerebral cortex subjected to RNAse T1 digestion (12.5 U/ml) for 30 min which produced an antisense hybridization signal identical to that observed without RNAse T1 digestion.

Antisense and sense hybridizations were also performed on liver and skeletal muscle. Both tissues have been reported not to contain detectable CNTF mRNA when assessed by Northern blot analysis of a total RNA extract (Stockli et al., 1989; Dobrea et al., 1992). However, selection of a true negative control was difficult to establish for CNTF, since many tissues have been reported to contain CNTF "activity" when extracts were

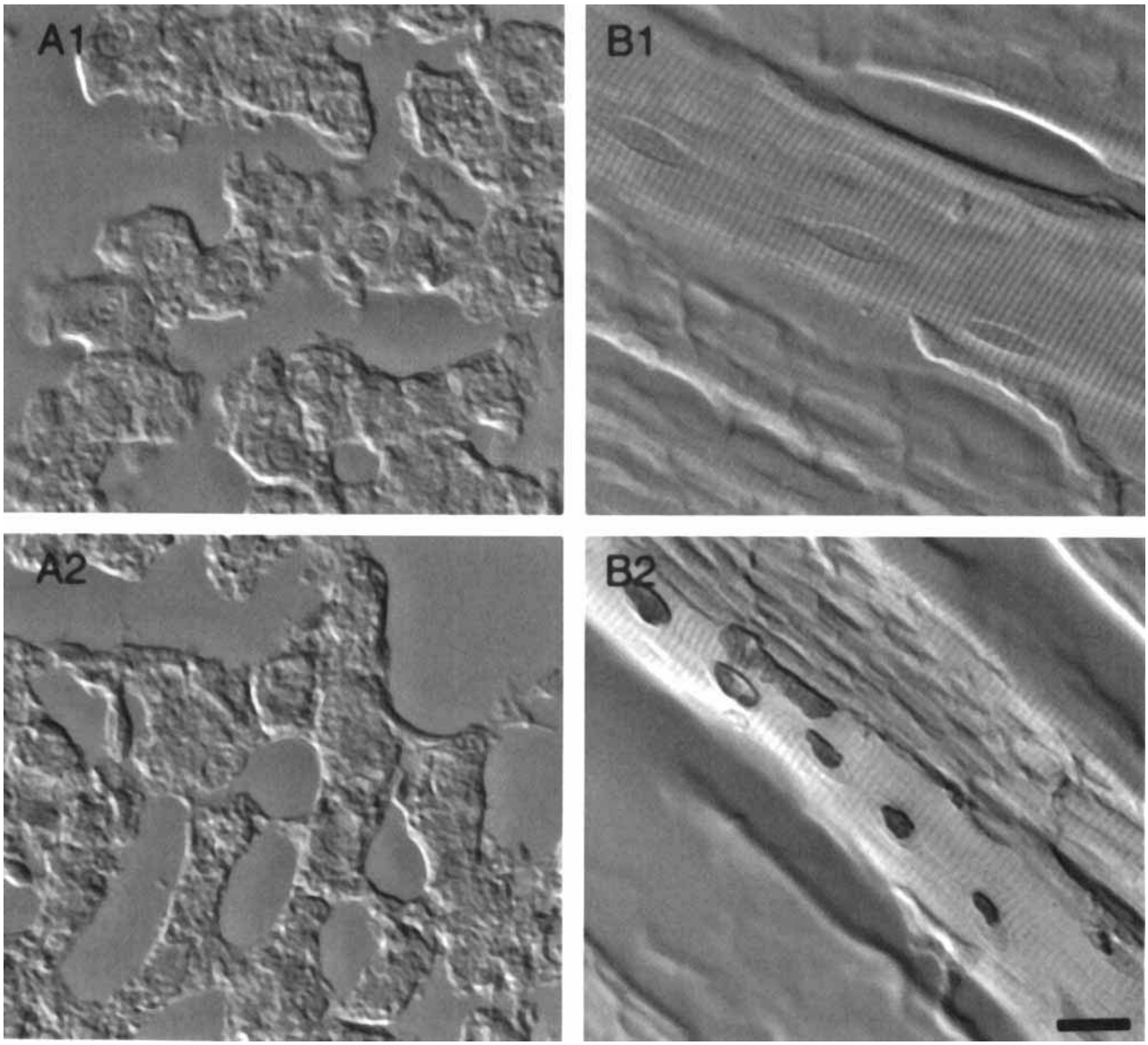


Fig. 4. Positive and negative controls for CNTF hybridization signal in non-neuronal tissue. **A1,A2:** Cryosections from adult rat liver. Hybridization with the CNTF sense probe revealed no signal (A1). Alternate sections hybridized with the CNTF antisense probe also revealed no detectable signal under conditions identical to that for rat CNS (A2). **B1,B2:** Cryosections from adult rat skeletal muscle. Hybridization with the CNTF sense probe revealed no signal (B1) while alternate sections hybridized with the CNTF antisense probe revealed a hybridization signal localized to the nuclei of muscle fiber cells (B2). Bar = 20 μm.

tested by bioassay, including liver and skeletal muscle (Manthorpe et al., 1993). The upper panels of Figure 4 illustrate high power, interference contrast micrographs of sense control probe hybridizations from cryosections of liver (Fig. 4A1) and skeletal muscle (Fig. 4B1). No signal was detectable in sense control probe hybridizations in either tissue. The lower panels of Figure 4 illustrate antisense hybridizations for liver (Fig. 4A2) and skeletal muscle (Fig. 4B2). No CNTF mRNA was detectable in the liver, but CNTF mRNA was clearly detectable in the nuclei of muscle fiber cells.

Sense probe hybridizations were used as a control for background signal due to hybridization with DNA and were performed on alternate serial sections, run simultaneously with each antisense hybridization. There was no detectable signal observed with the sense probe

hybridizations from any brain region for each developmental time point. CNTF sense probe in situ hybridizations are shown in the upper panels of Figure 5 for PD5 (Fig. 5A1), PD15 (Fig. 5B1), and adult rats (Fig. 5C1) in sections through the cerebellum at the level of the facial nucleus. Antisense hybridizations through the cerebellum of PD5 (Fig. 5A2), PD15 (Fig. 5B2), and adult rats (Fig. 5C2) demonstrate CNTF mRNA in most but not all cells. We had previously determined that CNTF immunoreactivity could be found in neurons and glia of the adult rat brain. Incubation of alternate sections with CNTF antibody revealed that CNTF immunoreactivity corresponded to that of the mRNA, and that there was apparently greater immunoreaction density in the nucleus compared with the cytoplasm. The lower panels of Figure 5 demonstrate CNTF immunoreactivity in neurons from PD5 (Fig. 5A3), PD15 (Fig. 5B3), and adult rat (Fig. 5C3) brain.

To confirm that CNTF mRNA was indeed localized to neurons, we performed a double label in situ hybridization with immunocytochemical staining against a neurofilament protein (using a polyclonal antibody that recognized both phosphorylated and non-phosphorylated forms). Images of double labeled facial motoneurons are shown in Figure 6. Figure 6A1 depicts a bright cytoplasm representing neurofilament antibody as localized by Vector Red under UV excitation with a rhodamine sensitive filter. Note the lack of signal in the nucleus. Since Vector Red and DAB reaction products are both visible with brightfield illumination, selective filtering was necessary to eliminate the red signal contributed by Vector Red. However, this filter method also decreased the visible DAB signal since it removed that portion contributed by red light wavelengths. The remaining DAB signal is depicted in Fig. 6A2, which demonstrates CNTF mRNA in both the cytoplasm and nucleus.

Figure 7 presents cortical neurons in sections taken from PD5 (Fig. 7A1), PD15 (Fig. 7B1), and adult rat (Fig. 7C1) brain. Note that again there was a strong nuclear signal in neurons at all ages and an apparent increase in the cortical neuron cytoplasmic signal with age. This appeared to be a common theme throughout the cortex independent of the rostral or caudal position of the section. The lower panels in Figure 7 demonstrate CNTF mRNA hybridization in cells within the oligodendrocyte lineage from the corpus collosum in PD5 (Fig. 7A2), PD15 (Fig. 7B2), and adult rat (Fig. 7C2) brain. Alternate sections examined with interference contrast optics demonstrated no GFAP immunoreactivity in cells of this morphology (data not shown), suggesting that oligodendrocytes express CNTF mRNA. In the PD5 oligodendroglia, CNTF mRNA signal strength is greatest in the cytoplasm exhibiting a perinuclear distribution while many cells have no detectable CNTF mRNA in their nuclei. By PD15, CNTF mRNA appeared to be more widely distributed throughout the cytoplasm and had increased in density. This pattern persisted in the adult CNS (Fig. 7C2).

Low power micrographs of CNS sections through the medial habenula and including CA1 hippocampal neurons and dentate gyrus are shown in Figure 8. A gradual increase in the intensity of CNTF mRNA hybridization signal is shown through PD5 (Fig. 8A), PD15 (Fig. 8B), and adult rat (Fig. 8C) brain. CNTF mRNA was detectable throughout the PD5 brain and the pattern of expression was analogous to that of the more mature PD15 and adult brains. CNTF mRNA was also apparent in the ependymal cells lining the cerebral ventricles in the glia limitans at all ages. This corresponded to the pattern of staining we previously reported using CNTF antibody in the adult rat brain. Although there was intense CNTF mRNA signal present in the medial habenula of the adult compared with cortical and hippocampal neurons, in general we found that cortical neurons presented a much stronger hybridization signal than many subcortical regions.

Cytoplasmic and nuclear OD measurements through randomly chosen facial motoneurons ($n = 30$) from PD5, PD15, and adult brain sections were used to quantitate CNTF mRNA hybridization signal with increasing age. To compare the nuclear and cytoplasmic signals, OD values were normalized to the mean of the cytoplasmic OD within each age group to provide a relative comparison. These values are shown in Figure 9, for facial motoneurons from PD5, PD15, and adult brain. The diagonal line represents a 1:1 ratio of relative ODs. Note that for each age group, the majority of nuclear OD values lie above the line. It was apparent that for most facial motoneurons in each age group, nuclear CNTF OD was greater than cytoplasmic OD. Furthermore, with increasing age, the nuclear:cytoplasmic relative OD decreased slightly and there was less variance of OD signal among individual neurons. In order to examine the relative expression of CNTF mRNA in facial motoneurons with increasing age, the relative cytoplasmic and nuclear ODs were normalized against the adult facial motoneuron cytoplasmic OD. These results are presented as box plots in Figure 10. An increase was found in the relative cytoplasmic OD with increasing postnatal age and this trend was also apparent for relative nuclear cytoplasmic OD values.

DISCUSSION

We have found that CNTF mRNA can be localized to both neurons and glia of rats as early as PD5 by in situ hybridization using biotin-UTP labeled antisense RNA probes. A greater accumulation of CNTF mRNA was

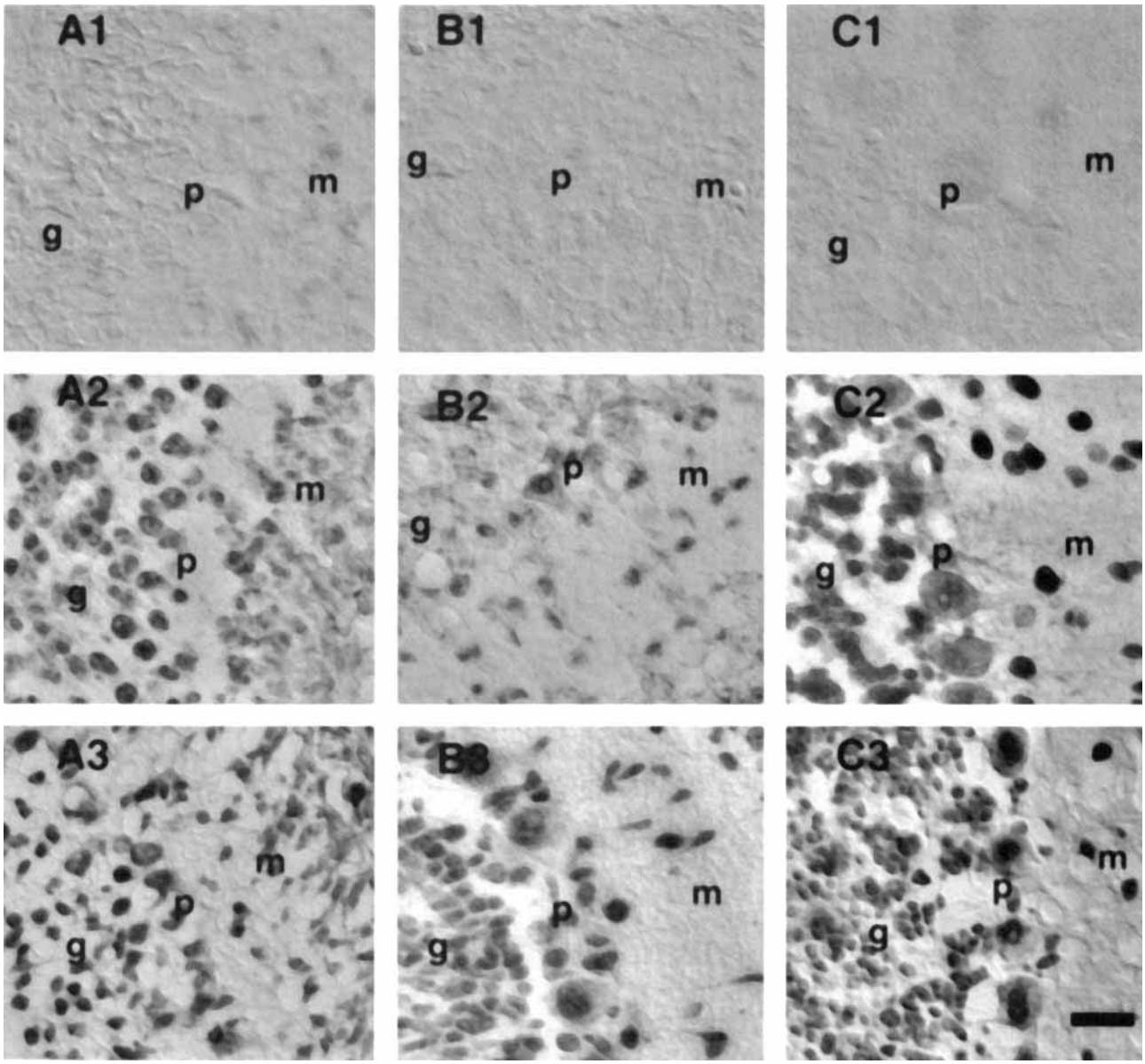


Fig. 5. CNTF protein and CNTF mRNA are expressed as early as PD5. The blank sense control probe hybridizations through the granule (g) cell, Purkinje (p) and molecular (m) cell layers of the cerebellum in PD5 **(A1)**, PD15 **(B1)**, and adult rats **(C1)**. Note that the sections display no detectable signal or at the most a faint immunoperoxidase reaction product in a few cells. Interference contrast optics were used to provide definition and visualization of the cell boundaries. Antisense probe hybridization revealed that CNTF mRNA was distributed throughout all cerebellar layers for PD5 **(A2)**, PD15 **(B2)**, and adult rat **(C2)** brain. **A3,B3,C3:** Alternate sections were immunoreacted with a polyclonal antibody against a CNTF peptide fragment. CNTF immunoreactivity was found in the brains of PD5 (A3), PD15 (B3), and adult rats (C3) and reflects the CNTF mRNA distribution depicted in A2, B2, and C2. Bar = 20 μm.

observed in the nucleus rather than the cytoplasm of most cells. Often, the nuclear distribution appeared as perinucleolar although both neurons and oligodendroglia were observed which did not contain significant amounts of CNTF mRNA in the nucleus. The subcellular distribution of CNTF mRNA was found to be analogous to that observed for CNTF immunoreactivity. This supports and extends our earlier finding that CNTF immunoreactivity was localized to both neurons and glia in the adult brain and spinal cord of rats and mice (Henderson et al., 1994). A gradual increase in the density of neuronal cytoplasmic and nuclear CNTF mRNA signal was evi-

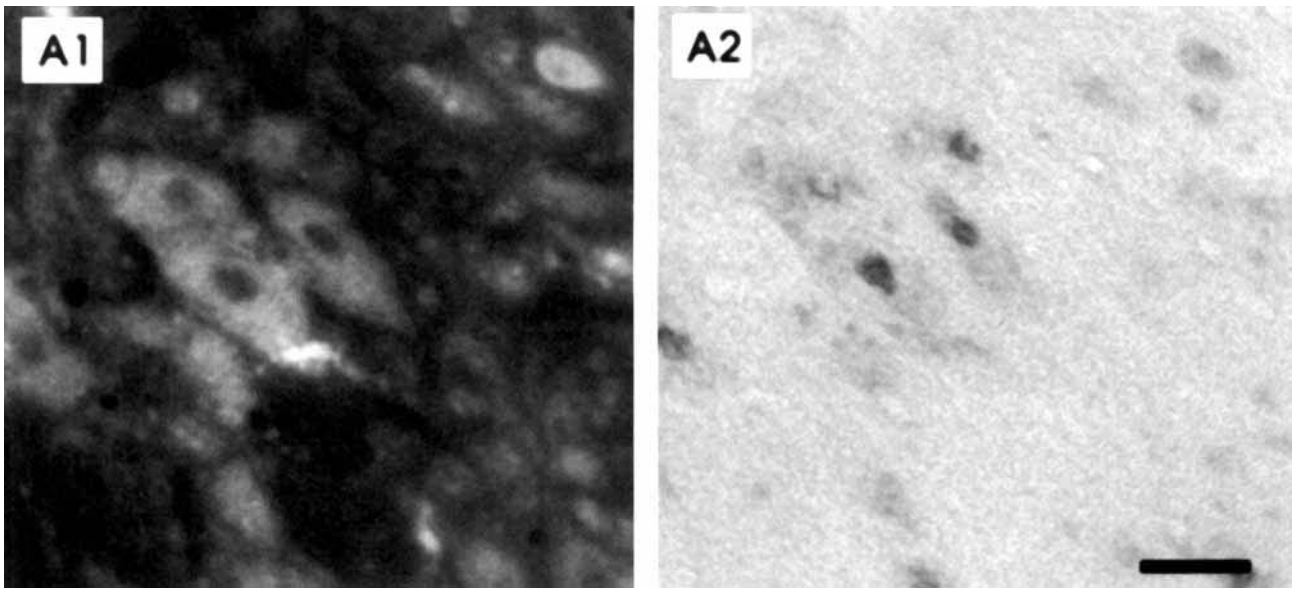


Fig. 6. CNTF mRNA and neurofilament antibody colocalized in facial motoneurons. Sections through adult facial motoneurons hybridized with a biotinylated riboprobe and then incubated with a polyclonal antibody to phosphorylated and unphosphorylated neurofilament. The antibody signal was visualized with Vector Red, which is detectable with both brightfield and UV excitation. **A1:** Neurofilament antibody visualized under UV excitation with a rhodamine sensitive filter demonstrating a bright cytoplasmic signal, but no nuclear signal. **A2:** The same neurons under filtered brightfield optics to eliminate the red brightfield signal of the neurofilament antibody. The remaining DAB signal representing CNTF mRNA is found in both the nucleus and cytoplasm of these facial motoneurons. Bar = 20 μm.

dent with increasing age. OD measurements of the CNTF hybridization signal in randomly chosen facial motoneurons demonstrated that for most facial motoneurons, the nuclear hybridization signal was greater than the cytoplasmic signal and that both increased over PD5 and PD15 to the adult stage. We also report that Northern blot analysis of poly(A)+ mRNA taken from freshly dissected adult rat cerebral cortex and brainstem (including di- and mesencephalon) revealed a readily detectable 4 kb transcript and a fainter 1.2 kb transcript when visualized with chemiluminescent detection.

Two different sized transcripts have been detected for CNTF mRNA on Northern analysis from rat CNS tissue, a 1.2 kb fragment (Stockli et al., 1989) and a larger 4 kb transcript from primary astrocyte and fibroblast cultures (Rudge et al., 1992). The larger transcript may be the result of an alternate polyadenylation site usage, since primer extension analysis has not revealed an alternate transcriptional start site (Carroll et al., 1993). CNTF does not possess a conventional signal peptide sequence and no secretory mechanism has been demonstrated to date, although it has been postulated that CNTF may be released by a novel mechanism. It has also been proposed that astroglia must be physically damaged in order to release CNTF so that the protein may exert its pleiotrophic actions on neurons (Ip et al., 1993; Manthorpe et al., 1993).

Our findings are in contrast to several previously published reports which have localized CNTF mRNA only to glial cells of the CNS and peripheral nervous system (PNS). An earlier study which examined CNTF mRNA by Northern blot analysis in the adult rat (Stockli et al., 1991) with total RNA extracts found detectable levels of CNTF transcript in those same brain regions in which we detected message by in situ hybridization. However, no blots were shown from these areas, and it is not known whether the authors detected the 1.2 kb fragment or the 4 kb fragment or both. A second study reported a 1.2 kb transcript in sciatic and optic nerves of the rat using oligonucleotide probes with a total RNA extract, but the brain was not examined (Dobrea et al., 1992). A 1.2 kb transcript was also found to be just detectable in a total RNA extract from whole adult rat brain (Rudge et al., 1992). Others have reported CNTF message in the CNS glia, but restricted their examination to primary "type 1" astrocyte cultures (Lillien and Raff, 1990; Carroll et al., 1993). Rudge and colleagues (1992) detected both a 4 kb and a 1.2 kb transcript in primary astrocyte and fibroblast cultures from the CNS. In addition, they reported a detectable CNTF mRNA (1.2 kb) transcript in cultured hippocampal neurons originating from the E18 rat after 4 days in vitro (less than 5% of the cells were non-neuronal). We have found that the 4 kb transcript is readily detectable in poly(A)+ extracts, but

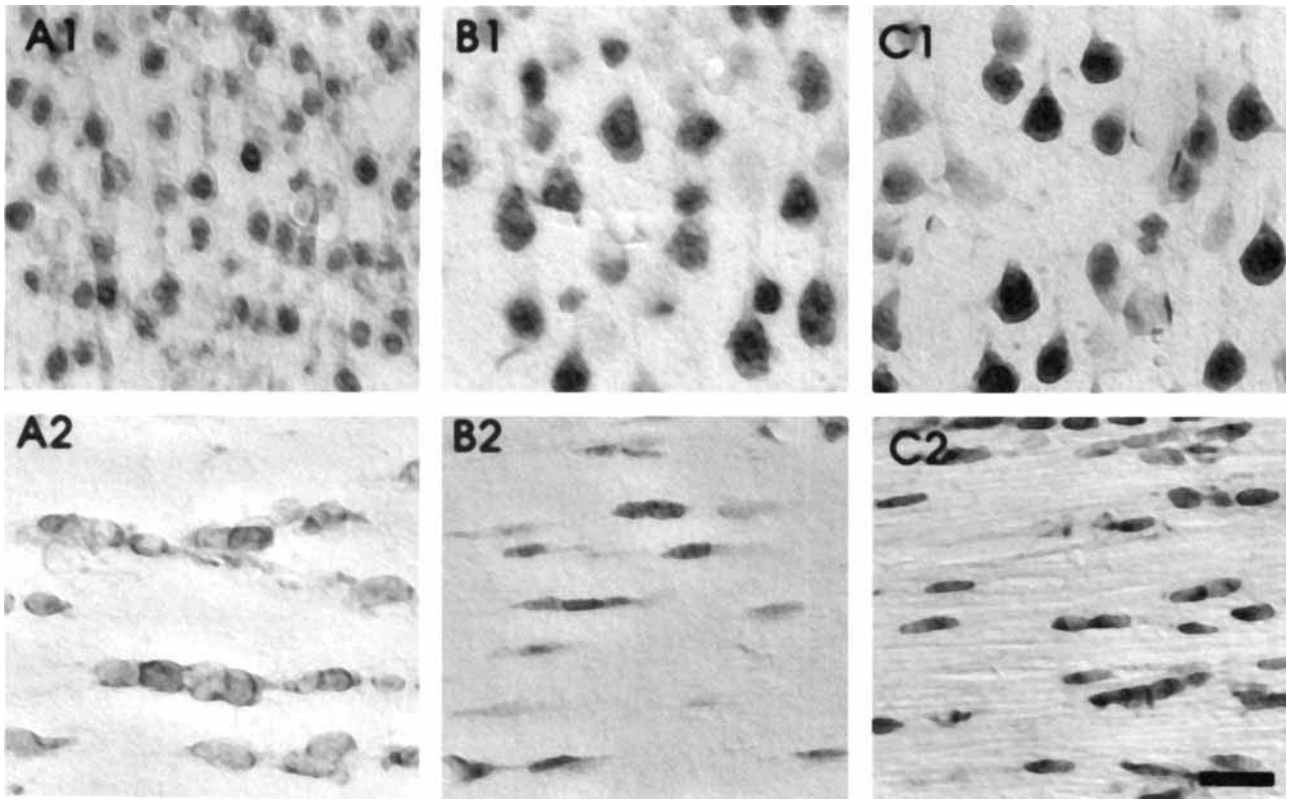
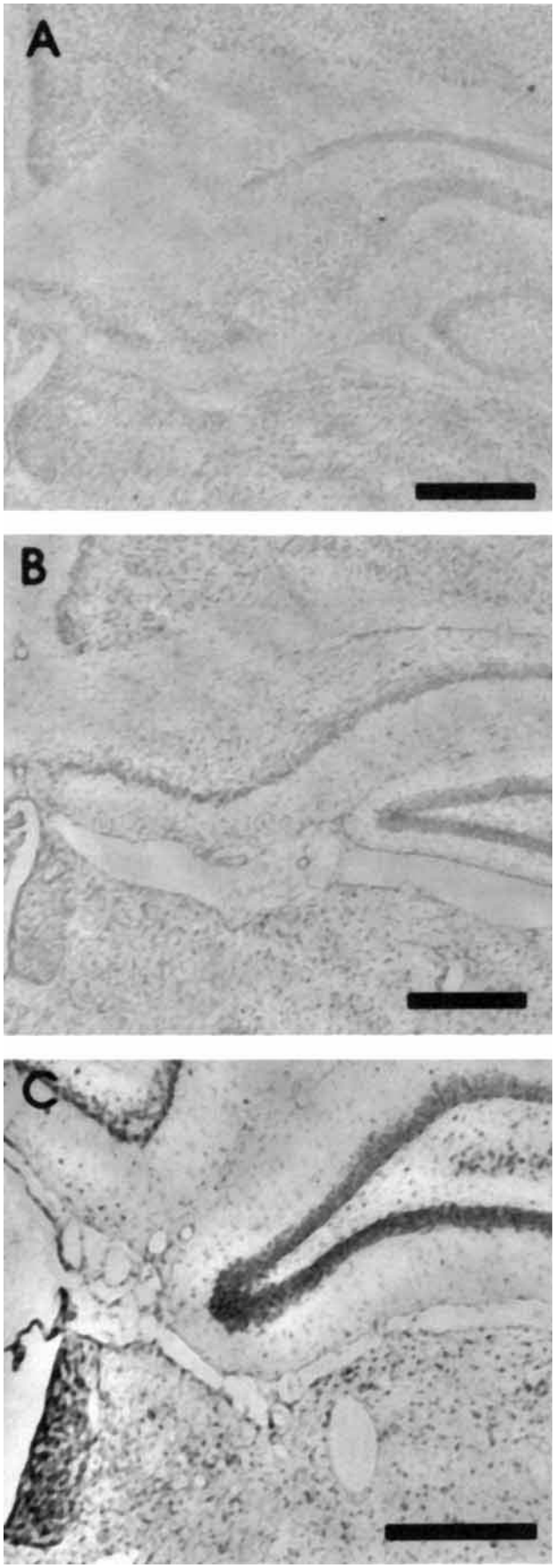


Fig. 7. Postnatal distribution of CNTF mRNA in cortical neurons and glia of the corpus collosum. CNTF antisense probe hybridizations in the cortex of PD5 **(A1)**, PD15 **(B1)**, and adult **(C1)** rat brain. At each age, the CNTF mRNA hybridization signal predominates in the nucleus of the cortical neurons. The density of the signal appears to increase with age. CNTF antisense hybridizations in the corpus callosum of PD5 **(A2)**, PD15 **(B2)**, and adult **(C2)** rats. The cells were GFAP negative (not shown), suggesting that they are oligodendroglia. A predominant cytoplasmic distribution of CNTF mRNA was observed in these cells. Bar = 20 μm.

the 1.2 kb extract is only just visible by chemiluminescent detection in the cerebral cortex and the brainstem. To date, there is no other published report which has examined CNTF message in the CNS using poly(A)+ extracts for Northern blot analysis. Examination of E18 embryonic brain by polymerase chain reaction (PCR) amplification did not detect any prenatal CNTF mRNA in vivo (Stockli et al., 1991).

Studies examining CNTF mRNA in the CNS by in situ hybridization have been limited to reactive astroglia in vitro (Seniuk et al., 1994) or reactive astroglia in vivo (Ip et al., 1993). Ip and colleagues (1993) did not report any CNTF message detectable in neurons; however, we would argue that the pattern of hybridization signal revealed in their low power micrograph through the hippocampus/midbrain is similar to the pattern displayed in Figure 8 of this manuscript, indicating a positive neuronal signal in the midbrain, hippocampus, and dentate gyrus. Unfortunately, higher power photomicrographs of regions other than the wound zone were not available for examination, nor a sense probe control, so it is not possible to determine whether ^{35}S probes would allow a definitive separation of a neuronal CNTF mRNA signal from a glial signal.

There have been few reports which examined the rat CNS for CNTF immunoreactivity. In some instances, it was only noted that no immunoreactivity was present (Dobrea et al., 1992), while in others detectable immunoreactivity was shown in primary type 1 cultures (Stockli et al., 1991; Rudge et al., 1992) or optic nerve (Dobrea et al., 1992) but other brain regions apparently were not examined. CNTF immunoreactivity has also been reported in Schwann cells, but the CNS was not examined in these studies (Friedman et al., 1992; Rende et al., 1992; Sendtner et al., 1992). In our hands, a polyclonal antibody against a peptide fragment of CNTF (residue numbers 131–147) revealed a relatively ubiquitous distribution for CNTF, similar to our localization of CNTF message by in situ hybridization. The specificity of the antibody was determined in part by Western blot



analysis which revealed a single band at approximately 23 kd in cytoplasmic extracts from rat sciatic nerve, primary astrocyte cultures, and C6 cells (for details see Henderson et al., 1994).

The use of biotin labeled riboprobes for in situ hybridization offers single cell (and subcellular) localization and rapidity of detection (the entire procedure can be completed in 1 day) compared to ^{35}S or ^{3}H labeled probes. Further, the use of antisense RNA probes can provide a considerably higher signal (compared to DNA probes) since there is no competition for hybridization to a complementary sense strand (Cox et al., 1984). Probe concentration for biotinylated riboprobes must be determined empirically, although recommended concentrations may vary from 200 ng/ml of hybridization solution to 2 mg/ml (Cox et al., 1984; Unger et al., 1991; McQuaid and Allan, 1992). Singer and colleagues (1987) determined that at high probe concentrations (10 mg/ml for a DNA probe) the extent of hybridization is much greater, while the signal to noise ratio is as good or better than that determined for low concentrations of probe. It has also been shown that higher RNA probe concentrations (up to 2 mg/ml) produce greater absolute signal and that although hybridization is mostly complete by 6 hr (Cox et al., 1984) a slower, second hybridization phase occurs over the next 12–18 hr (Angerer et al., 1987b). In our own experience (Seniuk et al., 1991, 1994) we generally begin with a probe concentration of 1000 ng/ml and then perform a dilution series to determine the optimal signal to noise ratio for the specific probe being tested. For most probes used at a high concentration we have found little difference in signal strength between a 4 hr hybridization and an overnight hybridization. Stringency washes and hybridization conditions were determined in part by examination of sense probe hybridizations (which must display no signal) run in parallel with antisense hybridizations and calculation of the melting temperature (Tm) value with 50% formamide. Generally, we obtained better morphology with biotinylated probes when hybridization temperatures were kept below 50°C and higher temperature washes were used to define stringency.

Northern blot analysis of poly(A)+ RNA from the adult cortex and brainstem resulted in a readily detectable transcript of approximately 4 kb in size and a just detectable fragment at 1.2 kb with 5 μg of poly(A)+.

Fig. 8. Increased CNTF mRNA expression with age. Low power photomicrographs of coronal sections taken through the medial habenula including the hippocampus in the diencephalon of PD5 **(A)**, PD15 **(B)**, and adult **(C)** rat brain. Note that the pattern of CNTF mRNA localization is identical for all ages and that CNTF mRNA can also be found in the ependymal cells lining the cerebral ventricles. There is an apparent increase in density of the hybridization signal with increasing age. Bar = 50 μm.

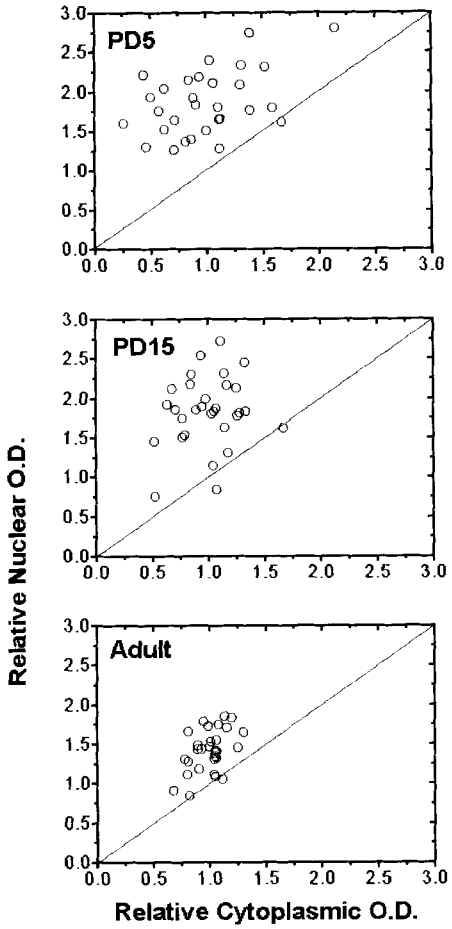


Fig. 9. Comparison of nuclear vs. relative cytoplasmic OD for PD5, PD15, and adult facial motoneurons. Nuclear and cytoplasmic OD values taken from randomly chosen facial motoneurons (n = 30) were background corrected (see Materials and Methods). To compare nuclear with cytoplasmic OD, the corrected values were normalized to the mean of the cytoplasmic OD within each age group. The diagonal line represents a 1:1 ratio of nuclear:cytoplasmic OD. All points above this line represent a greater nuclear CNTF OD relative to CNTF cytoplasmic OD. Note that for all age groups, nuclear CNTF mRNA OD was greater than the cytoplasmic OD. With increasing age, the relative nuclear:cytoplasmic OD decreased slightly as did the variance of OD signal among neurons within the age group.

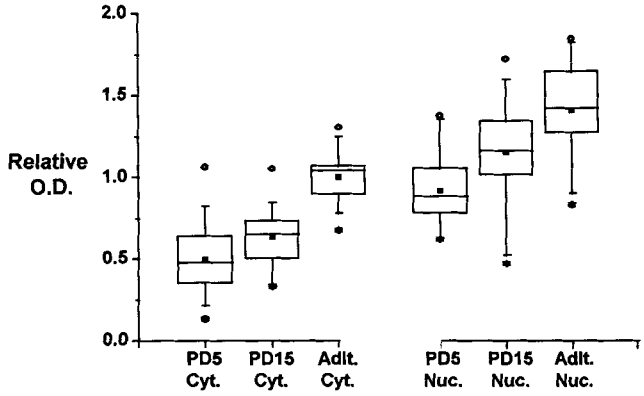


Fig. 10. Relative CNTF mRNA OD with increasing age relative to adult facial motoneurons. The relative nuclear and cytoplasmic OD values for randomly chosen facial motoneurons (n = 30) from each group were normalized against the mean adult cytoplasmic OD in order to examine the relative expression of CNTF mRNA with increasing age. These data are displayed as box plots. The box alone comprises 50% of the distribution of OD values, while the median is indicated by the bar and the mean by the black square within the box. The lower and upper error bars include the range of OD values from 10 to 90%. The points plotted above and below each box represent the maximum and minimum values. Note that there is a trend toward increased CNTF mRNA in both the nucleus and cytoplasm of facial motoneurons with increasing age.

The two transcript sizes may reflect glial and neuronal compartmentalization or the 4 kb transcript may be the primary polyadenylated form while the smaller transcript is not. Alternately, the 4 kb transcript may be the most abundant message in the adult brain, and therefore representative of a majority of postmitotic cells in the adult brain.

It is worth noting that previous examination of CNTF mRNA in the rat CNS (optic nerve, olfactory bulb) has been performed on total RNA extracts, resulting in detection of a 1.2 kb fragment. Rudge and colleagues (1992) found that a 4 kb transcript was just detectable from a total RNA extract from primary astrocytes in vitro. Interestingly, a poly(A)+ extract of rabbit sciatic nerve exhibited a transcript of approximately 4 kb in size, while total RNA extracts of rat sciatic nerve produced a 1.2 kb fragment. Previous studies which have examined the complexity of rat brain RNA by saturation hybridization, primarily a measure of the diversity of high complexity rare RNAs, have demonstrated no overlap between poly(A)+ and poly(A)− populations. Yet, in vitro translation has indicated that translation products directed by the poly(A)− fraction could also be found among the poly(A)+ translation products (Chikaraishi, 1979). It is conceivable, therefore, that both CNTF mRNA transcripts could be translated.

Like CNTF, acidic and basic fibroblast growth factors (aFGF, bFGF) both demonstrate nuclear localization by immunocytochemistry. It has been postulated that alternative initiation of translation can regulate the subcellular localization of bFGF (Bugler et al., 1991) and that translocation of bFGF to the nucleus is G1 phase cell cycle specific and appears to have a direct action on the level of ribosomal gene transcription (Bouche et al.,

1987; Baldin et al., 1990). More recently, it has been shown that increased expression of aFGF in quiescent cells functions as a cell survival factor rather than a mitogenic factor (Renaud et al., 1994). Exogenously administered aFGF can promote cell survival as well as control cell differentiation. It has been postulated that aFGF may not directly protect against cell death but may influence expression of genes involved in the death process. Interestingly, it appears that exogenously administered aFGF is translocated to the nucleus via a receptor-dependent process (Imamura et al., 1990) while the endogenous aFGF does not translocate to the nucleus (Zhan et al., 1992). Like aFGF, CNTF can also promote cell survival and differentiation. Our findings do not rule out the possibility that some novel release mechanism exists to provide astroglially derived CNTF to neurons nor the fact that paracrine induction by CNTF of neuronal genes may be achieved by this route. It is worth noting that the CNTF alpha receptor can be detected by Northern blot analysis in all brain regions in which we have detected CNTF mRNA by in situ hybridization. The CNTF alpha receptor exists in soluble form and has been shown to facilitate the action of CNTF (Davis et al., 1993). More recently, it has been reported that the Jak-Tyk family of cytoplasmic tyrosine kinases can associate with gp130 and LIF b receptor components in the absence of ligand, and that the addition of CNTF can elicit a distinct profile of Jak-Tyk phosphorylation in different cell lines (Stahl et al., 1994).

Neurons in early postnatal development are considered to be dependent upon target-derived trophic factor support for their survival (Lowrie and Vrbova, 1992). Normally, less than 30% of motoneurons survive separation from their target muscle prior to PD14 (Crews and Wigston, 1990). In contrast, axotomy of adult facial motoneurons results in approximately 70–95% survival (Snider and Thanedar, 1989; Pollin et al., 1991). The difference in survival is suggested to be contingent upon alternate sources of trophic support available to adult motoneurons. These would include mature, differentiated astroglia (Dutton, 1993), Schwann cells (Taniuchi et al., 1988), and afferent input. Although, the CNTF "knockout" transgenic mouse appears normal postnatally, after approximately 8 weeks, facial motoneurons begin to degenerate spontaneously (Masu et al., 1993), suggesting that endogenous CNTF may be important in the long-term survival of quiescent cells rather than determining the fate of immature neurons. This may occur either as a result of increased expression of nuclear CNTF, similar to aFGF, or CNTF may play a role in the consolidation of synaptic connections of motoneurons with their targets to ensure functional neuromuscular synapses. The development of functional neuromuscular synapses is ongoing during the first few postnatal weeks (Diamond and Miledi, 1962). It is possible that lack of CNTF during this critical development time window could result in neuron/muscle connections which may not provide adequate trophic support to facial motoneurons and/or appropriate signals which assure the complete morphological and functional development of the neuromuscular junction. Alternately, endogenous CNTF may be a requirement for postmitotic neuron survival.

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