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Localization of CNTF immunoreactivity to neurons and astroglia in the CNS

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Species specific antibodies were raised to a peptide of rat ciliary neurotrophic factor (CNTF – amino acids number 131–147). Following affinity purification, these antibodies were used to determine the pattern of CNTF immunoreactivity in adult rat and mouse brain, spinal cord, and sciatic nerve. Alternate sections stained using neurofilament and the affinity purified anti-CNTF antibody (HARC-1) demonstrate that CNTF immunoreactive neurons are present within the facial nucleus, dentate gyrus, olfactory bulb, basal forebrain, locus coeruleus, cortex and substantia nigra. In addition, neurons throughout the hippocampus, and Purkinje cells within the cerebellum also exhibit CNTF immunoreactivity. CNTF immunopositive neurons demonstrate a preponderance of nuclear staining, with some staining present in the cytoplasm. Alternate sections incubated with glial fibrillary acidic protein (GFAP) antibody also demonstrate glia which are positive for CNTF. In the peripheral nervous system, Schwann cells of the sciatic nerve exhibit strong immunoreactivity for CNTF, however staining is confined to the cytoplasm and is absent from the cell nucleus. These data demonstrate that CNTF immunoreactivity is broadly distributed throughout neurons and glia of the adult rodent nervous system.

INTRODUCTION

Ciliary neurotrophic factor is a 23 kDa acidic protein, initially identified by its in vitro trophic activities on chick ciliary neurons. Subsequently, CNTF has been shown to promote the survival of sensory, sympathetic, parasympathetic and motor neurons[9,25,27] *in vitro*. In vivo, CNTF has been shown to prevent the 'programmed' death of spinal cord motoneurons[25] and oligodendrocytes[24]; as well as the axotomy-induced death of motoneurons in the facial nucleus[34]. The axotomy induced death of cholinergic and non-cholinergic neurons of the medial septum has also been shown to be largely prevented through application of CNTF[14]. In addition, recent work has suggested that CNTF can reduce the degree of motoneuron axonal loss and improve survival in *pmn*, an inherited murine model of motoneuron disease[35]. With respect to cellular differentiation, CNTF has been shown to play a role in the conversion of O-2A progenitors derived from the optic nerve and the telencephalon, to an astroglial phenotype in vitro[21,23]; and to promote the cholinergic differentiation of sympatho-adrenergic precursors [31].

This trophic factor shares structural and morphogenic properties with members of the hematopoietic cytokines which include leukemia inhibitory factor (LIF), interleukin 6 (IL-6), granulocyte-colony stimulating factor and oncostatin M[4,27]. Glycosylation or other post-translational modifications of CNTF are not required for biological activity, the protein contains no known signal sequence, and does not appear to be secreted under normal conditions[12].

The actions of CNTF on its cellular target appears to be mediated via a tripartite receptor system which includes CNTFRα, LIFRβ and gp 130[6,38,40]. It is the α component which is thought to provide the dominant ligand binding domain for CNTF[7,17]. In situ hybridizations for the α receptor indicate that its expression is fairly ubiquitous in the adult rodent central nervous system (CNS); with prominent hybridization observed in the hippocampus, dentate gyrus, substantia nigra

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reticulata, subiculum, trapezoid body, Purkinje and granule cell layers and the sub-ependymal layer of the of the lateral ventricles[18]. In addition, motoneurons of the facial and trigeminal nuclei and those in layer V of the cortex, were also observed to exhibit substantial levels of CNTF α receptor. These regions (as well as a variety of others) also express LIFRβ and gp 130[17,18,40]. Thus a wide array of cells in the CNS appear to have the potential to respond to CNTF.

CNTF is expressed predominantly in Schwann cells in the peripheral nervous system and evidence suggests that this expression may be dependent upon axonal contact[10]. Consistent with this, axotomy of the sciatic nerve results in a dramatic decrease in CNTF mRNA distal to the lesion for periods of at least 1 month[36].

Within the CNS, expression of CNTF has been shown to occur in type I astrocytes in vitro[22]. In situ hybridizations using an antisense cDNA probe have demonstrated some region-specific distribution of CNTF mRNA, with spinal cord, brainstem and hindbrain showing higher levels of expression than regions such as the telencephalon[39]. In addition, sub-populations of GFAP positive astrocytes within the optic nerve also appear to contain CNTF[8,39]. As in the peripheral nervous system, the expression of CNTF appears to occur largely in the postnatal period[39]. However, to date there have been no systematic reports on the distribution of CNTF at the cellular level in the CNS. We therefore produced anti-peptide CNTF antibodies in order to localize the distribution of CNTF protein at the cellular level in vivo.

MATERIALS AND METHODS

Production of anti-CNTF antibody

The rat CNTF polypeptide EQKIPENEADGMPATVG (amino acids number 131–147) was synthesized on an ABI model 431A peptide synthesizer, using standard FMoc chemistry. Analysis of this peptide sequence was checked against all known protein sequences of any species using the Genetic Computer Group (GCG) software FastA program against the Swiss-Prot and PIR databases. The results indicate the homologies shown below.

The rat dihydropyridine-sensitive channel subunit represents the closest non-CNTF antigen with respect to the parent peptide. The antisera raised does not recognize the human CNTF species, indicating at it is quite specific for rodent CNTF. Thus, it is highly unlikely that this antisera recognizes the calcium channel subunit which exhibits a much lower degree of homology to the parent peptide and for which the majority of the amino acids composing this homology are shared with the human CNTF species. In addition, the known distribution of dihydropyridine-sensitive calcium channels (outer plasma membrane) does not correspond to the observed antigen distribution (nuclear and cytoplasmic). The peptide used to raise these antisera thus does not share significant homology with other known protein sequences.

Following cleavage, the peptide was purified by reverse-phase HPLC, using a C18 column. The product was then lyophilized and redissolved in a buffer of 100 mM NaCl, 20 mM sodium phosphate, pH 7.5. This solution was then extensively dialyzed against several changes of buffer, and stored at −20°C for latter use. Keyhole limpet hemocyanin (KLH) (Calbiochem) was dissolved and subsequently dialyzed (10,000 MW cutoff) against several changes of 10 mM $NaPO_4$, 160 mM NaCl, pH 7.2 (PBS) for 48 hours at 4°C. This solution was then centrifuged at 8,000 × *g* for 10 min. The resulting supernatant was then used for peptide cross-linking. Six mg of CNTF peptide were linked to 1 mg KLH in a 0.1% solution of glutaraldehyde in PBS. Under these conditions, only the terminal amino group and epsilon amino group of lysine are favored reaction products with KLH. The reaction was allowed to proceed for one hour at 25°C, at which time it was terminated by addition of 1 M glycine, pH 7.2, to a final concentration of 200 mM. The amount of CNTF bound was determined from the residual free CNTF peptide concentration. The KLH-peptide product was then dialyzed against several changes of PBS for 48 h at 4°C.

New Zealand White rabbits were individually pre-bled, and their serum isolated by standard methods[16]. This constituted the 'pre-immune' sera for each animal. Three days latter, each animal received 500 μl of a 50:50 emulsion of KLH-linked peptide (250 μg CNTF) and Freund's complete adjuvant, given as a set of injections directly into the popliteal lymph nodes. Each animal received three subsequent booster injections intra-muscularly at one month intervals, which consisted of 200 μl KLH-peptide (200 μg CNTF) emulsified with an equal volume Freund's incomplete adjuvant. Test bleeds were obtained 7–10 day following booster injections. Sera was then analyzed for anti-CNTF antibodies by microtiter ELISA assay (see below). Those animals which demonstrated a high antibody titer were retained for subsequent antibody production. Sera from these bleeds was isolated following a one hour incubation at room temperature and overnight storage at 4°C to allow for clot contraction. Sera was stored at −70°C until used. This sera is subsequently referred to as 'crude immune serum'.

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Rat: CNTF
EQKIPENEADGMPATV  Homology: 100%     Immune recognition:      yes
||||||||||||||||
EQKIPENEADGMPATV

Human: CNTF
EQKIPENEADGMPATV  Homology : 75%     Immune recognition:      no
| |||  |||||||   |
EYKIPRNEADGMPINV

Rat: Dihydropyridine-sensitive calcium channel alpha-1 subunit
EQKIPENEADGMPATV  Homology : 43.7%
|   ||||  ||
EDIDPENEDEGMDEDK
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Affinity matrix coupling

Eleven mg of CNTF peptide were linked to 1 ml washed Biorad Affigel 15 under anhydrous conditions in DMSO. This reduces potential hydrolysis of the active ester which can occur in aqueous solution, allowing only the desired reaction to proceed. Reaction of the peptide primary amino groups (N-termini and epsilon lysine) with this matrix results in the displacement of *N*-hydroxysuccinamide and the formation of an amide bond between the peptide and matrix. Linking was performed in a volume of 2 ml, at 20°C for 2.5 h. The reaction was terminated by addition of 1 M ethanolamine (pH 8.09) to a final concentration of 75 mM. Quantitation of the remaining free peptide demonstrated a linkage efficiency of approximately 92%. The resulting product was then washed sequentially with a solution of 1 M NaCl, 20 mM Tris-HCl, pH 7.5, and 100 mM glycine-HCl, pH 2.5. The column was then washed extensively and stored (4°C) in a buffer of 100 mM NaCl, 20 mM Tris-HCl, pH 8.0, which contained 0.02% sodium azide. Immediately prior to use, each column was pre-equilibrated with 20 column volumes of 100 mM NaCl, 10 mM Tris-HCl, pH 7.5.

Purification of anti-CNTF antibody

Crude immune sera was diluted 1:10 in a solution of 10 mM Tris-HCl, pH 7.5. This solution was then slowly passed three times through the affinity matrix. The matrix was then washed with 20 volumes of 10 mM Tris-HCl, pH 7.5, and 20 volumes of 500 mM NaCl, 10 mM Tris, pH 7.5. Antibodies were eluted with a solution of 100 mM glycine, pH 2.5 into collection tubes which were pre-loaded with 1/10 volume 1 M Tris, pH 8.0. This solution represents 'affinity-purified sera' and was typically used at a dilution of 1:500. Columns were then washed with 20 volumes of 10 mM Tris, pH 8.8 and stripped with a solution of 100 mM triethylamine, pH 11.5. They were then sequentially re-equilibrated with 20 volumes of 100 mM and 10 mM Tris, pH 7.5. Elution at low pH was found to be more effective at removing functional anti-CNTF antibodies than high pH conditions. Typically 90% of anti-CNTF activity was removed following elution of 5 column volumes at low pH. More stringent washing of the column did not remove additional CNTF reactive material. With the exception of those used for ELISA analyses, affinity purified antibodies were stored in a solution of 1% BSA, 0.02% NaN_3 at 4°C until used.

ELISA assays

ELISA assays were performed as previously described[16]. Briefly, purified CNTF, CNTF peptide and control peptides were bound to PVC microtiter plates in a solution of phosphate-buffered saline (PBS) for 2 h at 25°C. Wells were then washed two times with PBS and blocked in a solution of 3% bovine serum albumin (BSA), 0.02% NaN_3, in PBS overnight. Wells were then washed twice in PBS and immune sera, diluted in blocking buffer, was then added at the appropriate dilution. After 4 h, plates were washed four times with PBS and incubated with alkaline phosphatase linked goat anti-rabbit (secondary) antibody, diluted 1:1500 in blocking solution. Following 2 h of incubation at room temperature, plates were washed four times in PBS and twice with 0.5 mM $MgCl_2$, 10 mM ethanolamine, pH 9.5. Wells were then reacted with a solution of 1 mg/ml p-nitrophenyl phosphate, 0.5 mM $MgCl_2$ 10 mM diethanolamine, pH 9.5 for thirty min at room temperature. Reactions were stopped with an equal volume of 0.1 M EDTA. Plates were then read at 405 nm.

Gel electrophoresis / Western analysis

Samples were typically resolved using a 15% polyacrylamide gel as previously described[3]. Samples for SDS–PAGE were placed in loading buffer of 2% 2-mercaptoethanol, 2% SDS, 40% glycerol, 125 mM Tris-HCl, pH 6.8; and boiled at 100°C for 5 min prior to loading on the gel. Gels were electrophoresed at 150 V for 3 h. Native gels were as those for SDS–PAGE but without the addition of SDS and without boiling of the samples prior to loading. A set of prelabelled dye-bound low MW standards (Biorad) were run with each sample set.

Following separation, proteins were transferred to nitrocellulose membranes (Schleicher and Schuell BA85) via electrophoretic transfer in a solution of 20% methanol, 150 mM glycine, 20 mM Tris, pH 8.0 at 40 V, 160 mA for 2 h. Membranes were then placed in a solution of 5% milk protein, 10 mM Tris, pH 8.0, 0.02% NaN_3 (blotto) at 25°C for 12 h. Primary antibody was then added for a period of 4–12 h at 25°C with mild agitation. Membranes were then washed in five changes of 0.05% Tween 20, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0 (wash buffer) for 5 min at room temperature. Secondary antibody (Promega alkaline phosphatase anti-rabbit) was then added at a dilution of 1:1500 in wash buffer, and incubated for 2 h at room temperature with mild agitation. Blots were then developed for 20 min, using a bromochloroindolyl phosphate/Nitro Blue tetrazolium substrate (0.0165% and 0.033%, respectively) in a solution of 5 mM $MgCl_2$, 100 mM NaCl, 100 mM Tris-HCl, pH 9.5. Reactions were stopped by rinsing the blots in PBS containing 20 mM EDTA, pH 8.0.

Immunocytochemistry

Adult rats (6 months) or mice (4–6 months) were perfused transcardially with either 4% paraformaldehyde (rat) or 2% paraformaldehyde/0.1% gluteraldehyde (mouse). These perfusion protocols were found to be optimal for each of the tissues examined. Brains with C_1–C_2 level spinal cord attached were removed in one piece. In addition, a portion of the sciatic nerve was taken and all tissues were given a 4 h post-fix in 4% paraformaldehyde at 4°C. Tissues were then put through several changes of cold 0.1 M PBS followed by cold 30% sucrose. Serial, coronal 10-μm-thick frozen sections were then taken through the brain and spinal cord, while 10-μm-thick longitudinal sections were taken through the sciatic nerve. Sections were rinsed in 0.1 M PBS, followed by 0.1 M glycine/0.1 M Tris (pH 7.0) for 30 min, followed by blocking with 10% normal goat serum (NGS) for 30 min. Sections were then reacted with affinity-purified anti-CNTF antibody (1:500 dilution of a 5 μg/ml stock, in 1% NGS/2% normal mouse serum/0.1 M PBS) overnight at 4°C. Alternate sections were Nissl-stained or incubated with either mouse monoclonal anti-GFAP (1:800 in 1% normal horse serum/0.1 M PBS) (Sigma, cat. no. G3893) or mouse monoclonal anti-phospho-neurofilament antibody (1:1000 in 1% normal horse serum/0.1 M PBS) (Sternberger Monoclonals Inc., no. SMI33) overnight at 4°C. Sections were then rinsed with 0.1 M PBS, and incubated with biotinylated IgG (1:200 dilution of goat anti-rabbit or horse anti-mouse) for 40 min at room temperature, rinsed briefly, then incubated with avidin–HRP (1:200 dilution of standard Elite kit, Vector Labs) for 30 min. Sections were then rinsed, and reacted with 1 mg/ml diaminobenzidene (Sigma) 0.02% v:v hydrogen peroxide to visualize the location of the antigen–antibody complexes.

Controls for CNTF immunoreactivity which included: secondary antibody alone, primary antibody alone, pre-immune rabbit serum plus secondary antibody, goat serum plus secondary antibody, and mouse serum plus secondary antibody, were all negative. Affinity-purified CNTF antibody which was used at a working concentration of approximately 0.1 ng/ml was adsorbed with pure rat CNTF peptide or pure rat CNTF protein (kindly provided by Dr. P. Richardson) at a ratio of 20:1 (peptide or protein:antibody). Sections incubated with this adsorbed antibody mixture were blank. Sections incubated with desorbed serum (depleted of anti-peptide antibody) from the affinity column eluate were also blank.

RESULTS

Specificity of anti-peptide antibody

In order to further define the pattern of CNTF distribution within the adult CNS, we synthesized a short 17 amino acid peptide derived from the rat CNTF sequence. The peptide EQKIPENEADGM-PATVG was chosen for synthesis because of its favorable hydropathic properties, its divergence rabbit and human CNTF isoforms as well as other from known

cytokine/neurokine members, its lack of strong conformational parameters and homology to other known protein sequences; and its ease of amino-linking chemistry. In addition, this sequence lies in the 'hinge region' between cytokine helices 'C' and 'D' as postulated by Bazan, and is immediately adjacent to the 'D1' motif within this molecule[4]. This region (D1) shares homology among several neurokine members, and antibodies which can bind adjacent to this site in the hinge region might, therefore, be expected to alter the functional properties of this molecule. It should be noted that subsequent to the synthesis of this peptide, a larger peptide which includes this sequence was shown to induce antibodies which recognize CNTF in frozen sections of sciatic nerve[39].

Western analysis of the pre-immune, immune, affinity-purified and peptide desorbed sera are shown in Fig. 1, panels A, B, C, and D respectively. The bands shown in Fig. 1A represent proteins recognized by the pre-immune serum antibodies present *prior to* inoculation with the CNTF-KLH complex, and thus do not exhibit reactivity to the CNTF present in the rat sciatic nerve extract in the right hand lane. As shown in Fig. 1B, following inoculation with the KLH–CNTF peptide complex, several additional bands are present, some of which represent protein recognition by CNTF anti-peptide antibodies. The sciatic nerve extract now exhibits immunoreactivity, most prominently at a band of approximately 23 kDa. Fig. 1C shows the result obtained with a blot identical to that shown in Fig. 1A,B, which has been incubated with the affinity-purified CNTF anti-peptide antibody (HARC-1), demonstrating the true pattern of CNTF specific immune recognition for each of the samples shown. By comparing Fig. 1B and 1C, it is apparent that affinity purification using the CNTF peptide removes the large number of non-CNTF recognizing antibodies present in the sera. These include antibodies which may recognize KLH and/or the glutaraldehyde cross-linking reagent due to differences in the linking chemistry of the affinity matrix.. Antibodies which do not recognize CNTF are present in the initial column eluent, and their immunoreactivity is shown in Fig. 1D. Note that while the affinity purified antibody is free of other contamination activities (Fig. 1C), the affinity column flow-through (Fig. 1D) contains a variety of different antibody activities, but does not contain appreciable amounts of CNTF anti-peptide activity (Fig. 1D), thus verifying the elution conditions.

Together these data demonstrate that immunization with the KLH linked CNTF peptide results in the development of anti-peptide antibodies which specifically recognize a protein of 23 kDa in extracts of sciatic nerve and purified (rat) type I astrocytes from cerebral cortex; consistent with previous observations of CNTF protein in these tissues[8,12,39]. The 23 kDa band actually consists of a doublet in these extracts and is also observed for recombinant CNTF purified from *E. coli.* Gupta et al. have previously shown that these bands represent CNTF isoforms which both contain high levels of CNTF specific activity[12]. An additional band of approximately 45 kDa is also observed in sciatic nerve extracts. Both the 23 kDa doublet and this 45 kDa CNTF isoform have now been observed, using *four* different antisera raised against rat CNTF derived from four independent laboratories. These include antisera raised against both the entire rat CNTF protein (ref. 10 and Richardson, see below), and rat CNTF peptides 45–59[29] and 131–147 (HARC-1). With respect to the 45 kDa band, it is interesting to note that Rende et al. have raised antibodies to two CNTF peptides, and that the higher molecular weight species was observed only with the higher affinity anti-peptide antisera[29].

As shown in Fig. 1, no CNTF specific immunoreactivity is observed for extracts prepared from either undifferentiated P-19 embryonal carcinoma cells or rat C6 glioma cells (ATCC CCL, no. 107) under these conditions (100 μg total protein/lane). Similar CNTF-specific results are obtained under non-denaturing conditions (data not shown). At higher protein concentrations we do observe some immunoreactivity with rat C6 glioma cells, consistent with the observation that these cells express low levels of CNTF[30].

The species specificity of this polyclonal antibody is shown in Fig. 2A. As can be seen from these data, sciatic nerve extracts probed with affinity purified anti-peptide antibody demonstrate a prominent immunoreactive band at 23 kDa for both rat and mouse, while similar extracts from human and rabbit do not exhibit immunoreactivity. The higher MW background observed with the rabbit extract represents non-specific reaction of the secondary (anti-rabbit IgG) antibody. The antigen specificity of HARC-1 is shown in Fig. 2B. Homogenates of various tissues derived from 28 day old CD1 mice show no immunoreactivity with extracts prepared from heart, liver, kidney, quadriceps muscle, spleen, or thymus under these conditions (150 μg total protein/lane), demonstrating the highly restricted pattern of expression of the epitope recognized by this polyclonal antibody. In fact, we have thus far observed immunoreactivity with this antibody only in native or reducing homogenates of rat and mouse brain, peripheral and optic nerve; and in cultures of purified type I astrocytes. Consistent with this, 10 μm frozen sections of liver, kidney or muscle stained for CNTF show no

specific immunoreactivity under the conditions used for this study (see Fig. 7). Fig. 2C demonstrates cytoplasmic and cell supernatant fractions of rat C6 glioma cells, or C6 lines which have been stably transfected with a complete rat CNTF cDNA. These stably transfected C6 lines produce either a secreted or non-secreted form of CNTF. These data confirm that the anti-peptide antibody raised specifically recognizes the rat and mouse species of CNTF.

To further insure the specificity of the affinity purified HARC-1 antisera, an additional test was performed. Cell and tissue extracts which were positive for CNTF with this antisera were analyzed with a second polyclonal CNTF antibody. This antisera (PS 8–4) was raised against the entire CNTF protein and demonstrates function-blocking activity in vitro (kindly provided by Dr. P. Richardson). This antisera demonstrates a pattern of immune recognition very similar to that of HARC-1 in blots identical to those shown in Fig. 2B,C. A comparison of these two antisera using identical samples of type 1 astrocytes, sciatic nerve, whole brain and CNTF-transfected C6 cell extracts, as well as recombinant rat CNTF are shown in Fig. 3A,B. An almost identical pattern of immunoreactivity is observed for the two antisera. An interesting exception is whole brain extract, in which PS 8–4 recognizes an additional species of higher molecular weight (approximately 40 kDa) which is not observed with HARC-1. This 40 kDa species is distinct from the higher molecular weight species observed by both antisera in extracts of sciatic nerve, recombinant CNTF and transfected C6 cells (45 kDa). The exact nature of the 40 kDa species observed using PS 8–4 is as yet undetermined. However it has so far only been ob-

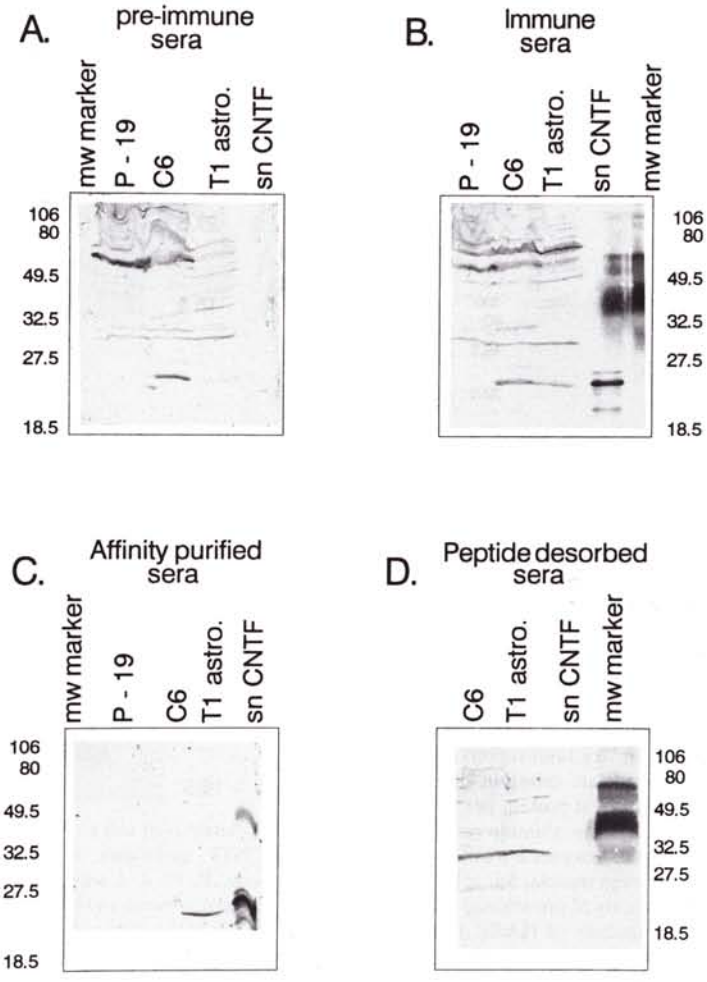


Fig. 1. Purification of HARC-1 antibody. Samples were electrophoresed on a 15% SDS–polyacrylamide gel for 3 h at 150 V (cv) then transferred to nitrocellulose for Western analysis. Identical samples consisting of 100 μg total protein per lane were loaded for the four sets shown. Protein samples were as follows: P-19, homogenate of P-19 embryonal carcinoma cell line (undifferentiated); C6, homogenate of rat C6 glioma cell line; T1 astro., purified culture of type 1 rat astrocytes from postnatal day 2 rat pups, following 10 days in culture; sn CNTF, purified adult rat sciatic nerve CNTF. Molecular weight markers are indicated in kilodaltons. The primary antibody was used at low dilution (1:100), in order to determine if contaminating activities were present for each condition shown. Western blots of identical samples are shown, developed with anti-rabbit IgG secondary antibody plus each of the following primary antibodies: (A) pre-immune sera, (B) crude immune sera, (C) affinity-purified sera, (D) peptide desorbed sera.

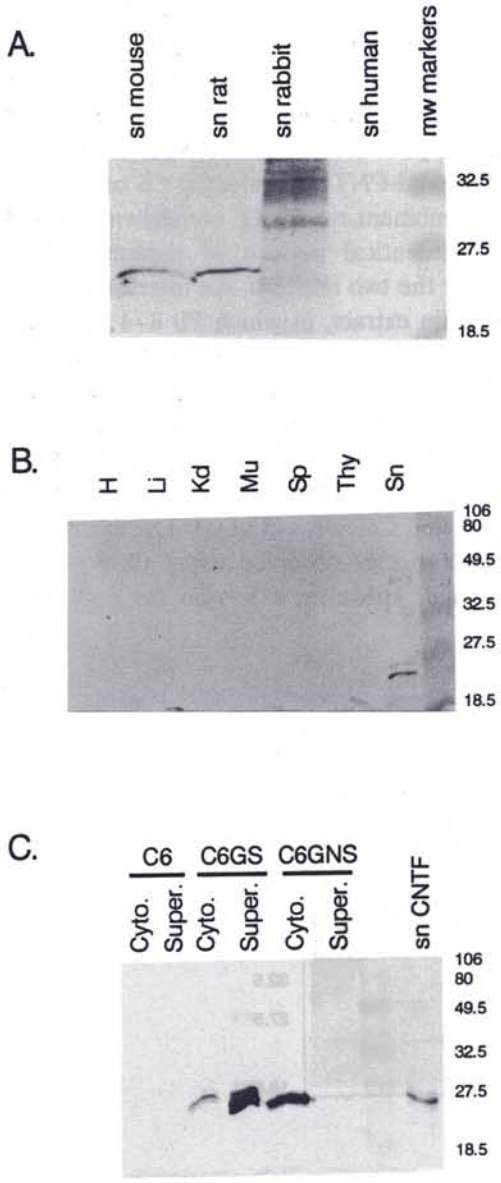


Fig. 2. Specificity of HARC-1 antibody. Samples were electrophoresed on a 15% SDS–polyacrylamide gel for 3 h at 150 V (cv), then transferred to nitrocellulose for Western analysis. A: species specificity of anti-peptide antibody. Each lane represents 50 μg total protein of a 10 K supernatant of homogenates of peripheral nerve from the indicated species. As shown in the figure, HARC-1 reacts with CNTF present in mouse and rat peripheral nerves, but not that from human or rabbit sources. Background in the rabbit lane is due to non-specific reactivity with the secondary (anti-rabbit) antibody. Molecular weights of pre-stained standards are shown in kilodaltons. B: tissue homogenates containing 150 μg total protein per lane were run under reducing conditions on a 15% polyacrylamide gel. Samples were prepared from 28-day-old CD1 mice. Samples are as follows: H, heart; Li, liver; Kd, kidney; Mu, quadriceps muscle; Sp, spleen; Thy, thymus; Sn, sciatic nerve. Molecular weights of pre-stained standards are shown in kilodaltons. C: immunoreactivity of HARC-1 antibody with cytoplasmic and cell-free supernatants of parental and transfected rat C6 glioma cell lines. Each lane represents the total extract obtained from 60,000 cells grown in serum free medium for 24 h. Lane descriptions are as follows: C6, parental C6 line; C6GS, C6 cell line transfected with a secreted form of rat CNTF; C6GNS, C6 line transfected with a non-secreted (naturally occurring) form of rat CNTF; cyto., homogenate of cell cytoplasm; Super, cell-free concentrate of extracellular medium; sn CNTF, purified rat sciatic nerve CNTF. Molecular weights of pre-stained standards are shown in kilodaltons.

served in extracts of rat and mouse brain, and can be competed using recombinant hCNTF. This 40 kDa species may therefore represent an alternate isoform of CNTF. Because our HARC-1 antisera recognizes only the lower molecular weight species previously observed for CNTF we believe that the staining pattern of this antisera gives a true representation of the localization of CNTF within the CNS. In addition, it is possible that these two species are co-localized within a given cell, as sections stained with these two antisera are remarkably similar with respect to their spatial patterns of immunoreactivity. Within a given CNTF-immunopositive (CNTF$^+$) cell, PS 8–4 appeared to stain the cell cytoplasm more intensely, while HARC-1 gave

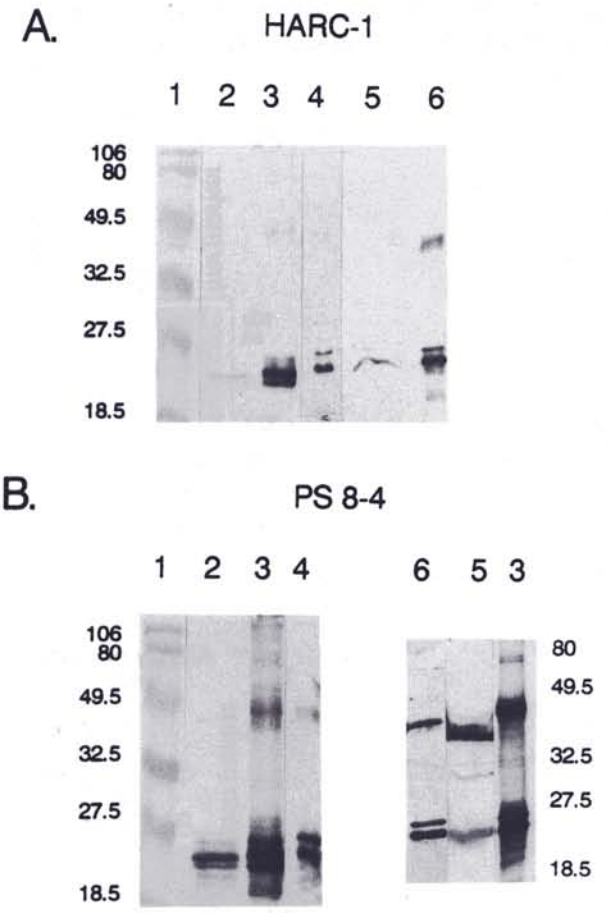


Fig. 3. Analysis of cell and tissue extracts using HARC-1 and PS 8–4 anti-CNTF antibodies. A: HARC-1 affinity purified anti-peptide antibody. B: PS 8–4 antibody raised against full-length CNTF protein. Samples shown in blots A and B represent identical extracts run on a 15% SDS–polyacrylamide gel. Samples and total protein content (Pierce BCA system) are as follows: (1) Pre-stained Bio-Rad low molecular weight standards, (2) 100 μg of a primary culture of 'type-1' astrocytes – rat (14 days in vitro), (3) 20 μg of C6 glial cells stably transfected with full-length rat CNTF cDNA, (4) 70 μg rat sciatic nerve extract, (5) 150 μg whole brain extract – rat, (6) 200 ng recombinant CNTF – rat. Color development for all blots shown was obtained using an alkaline phosphatase/NBT/BCIP system. The length of the color development period was 7 min for A, 15 min for B lanes 1–4 and 30 min for B, lanes 6, 5, 3. The molecular weight of the size standards is indicated in kilodaltons.

more nuclear staining in the majority of CNS cells, as indicated below.

Tissue localization of CNTF protein as visualized by immunocytochemistry

$CNTF^+$ neurons and glia were found to be widely distributed throughout the rat and mouse CNS, with most neurons in the rodent CNS exhibiting CNTF immunoreactivity using HARC-1. Positive neurons were observed in the cerebral cortices, the cerebellum, in subcortical regions as well as in the spinal cord. Typically neurons in the CNS demonstrated the pattern of immunoreactivity shown in Fig. 4A. In general, a greater intensity of immunoperoxidase staining was observed in the region of the cell nucleus compared with the cytoplasm of the neuron, suggesting a preferential subcellular localization of CNTF protein in the normal adult rodent.

A number of controls were included to ensure antibody specificity. Normally, sections were incubated sequentially with affinity purified primary antibody, secondary (biotinylated goat anti-rabbit IgG) antibody (2°), HRP-avidin (H-A), and diaminobenzidene (DAB). For the controls however, sections were incubated with the following combinations: (1) pre-immune serum followed by secondary antibody (2°), followed by H-A/DAB, (2) 2° followed by H-A/DAB, (3) primary antibody followed by H-A/DAB, (4) normal antibody diluent minus the primary antibody followed by 2°/H-A/DAB, (5) 2% mouse serum followed by 2°/H-A/DAB, (6) primary sera, desorbed of CNTF anti-peptide antibodies via affinity chromatography plus 2°/H-A/DAB, (7) affinity purified CNTF primary antibody adsorbed with pure CNTF peptide or (8) pure CNTF protein at a ratio of 20:1 (peptide or protein:antibody) followed by 2°/H-A/DAB. *In each case* (1–8), incubation of coronal sections with the above combinations resulted in no specific pattern of immunoreactivity. Results obtained with combinations (7) and (8) can be seen in Fig. 4B and 4C, respectively. The above results demonstrate that the staining obtained on tissue sections using the affinity purified anti-peptide antibody (as shown in Fig. 4A) is CNTF specific.

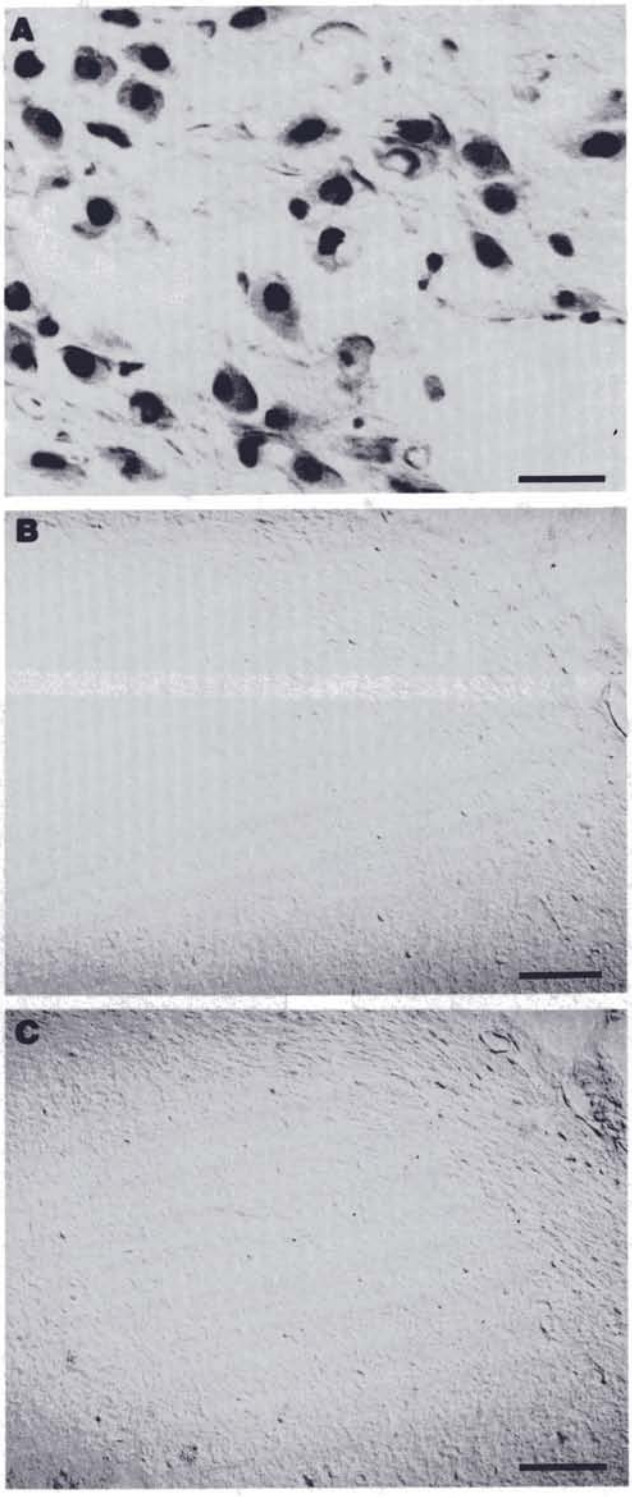
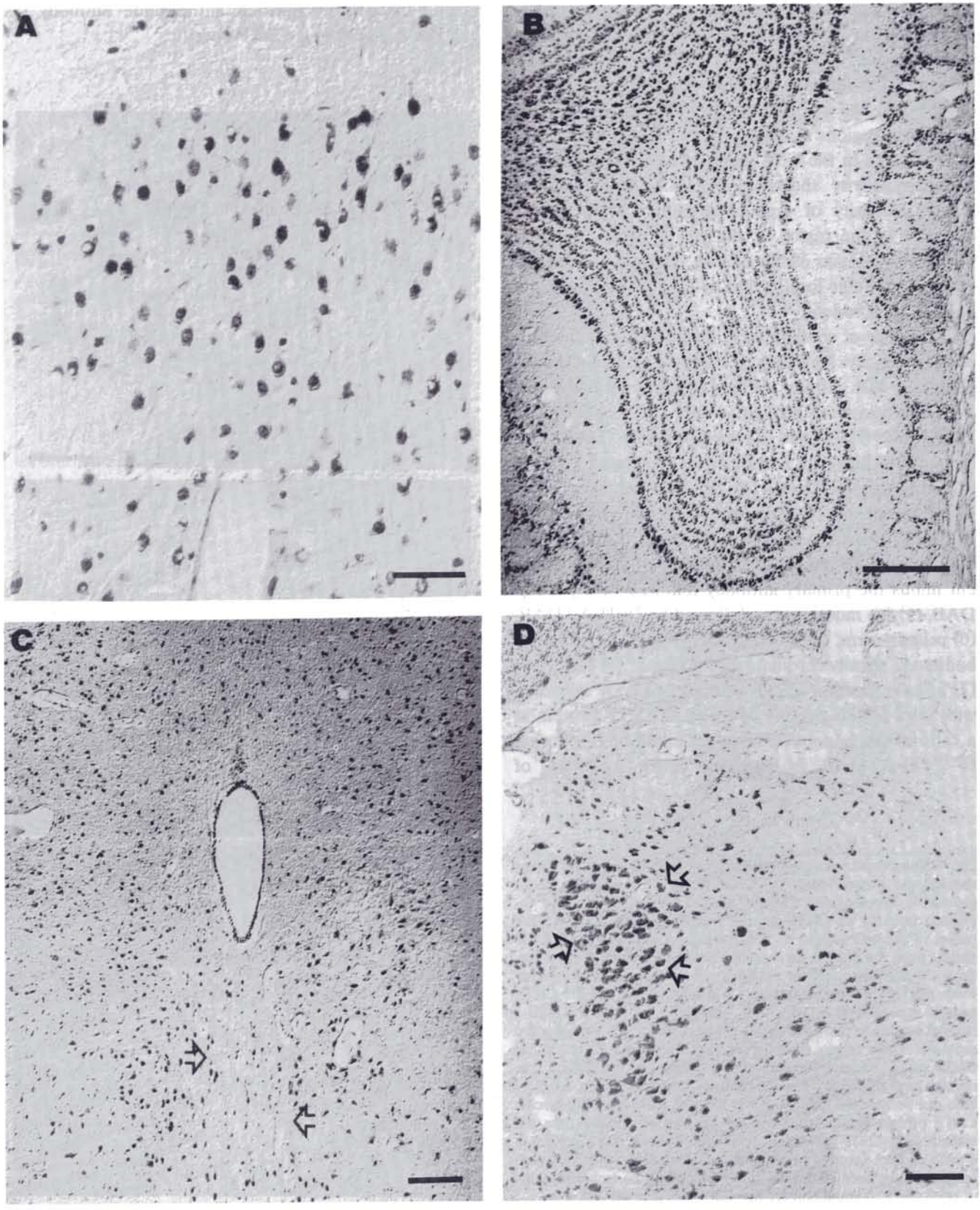


Fig. 4. Interference contrast photomicrographs of coronal sections through the normal adult rat CNS. A: $CNTF^+$ neurons in the supraoptic hypothalamic nucleus. This section illustrates the increased intensity of CNTF immunoreactivity observed within the region of the cell nucleus of the neuron as compared to the cytoplasm. Note the lack of non-specific background immunoreactivity. Bar = 30 μm. B: control for specificity of anti-peptide immunoreactivity. CNTF anti-peptide antibody was adsorbed to the rat CNTF peptide at a ratio of 20:1 (peptide:antibody), and incubated with a coronal section at the level of the olfactory bulb using the standard protocol. There is no evidence of specific immunoreactivity for CNTF using the adsorbed antibody complex. For comparison, the normal level of CNTF immunoreactivity observed in the olfactory bulb can be seen on Fig. 5B. Bar = 100 μm. C: control for specific CNTF protein immunoreactivity. CNTF peptide antibody was adsorbed to rat CNTF protein (P. Richardson, sciatic nerve extract) in a ratio of 20:1 (protein to antibody) and incubated with a coronal sections at the level of the olfactory bulb. Note the lack of specific immunoreactivity. Bar = 100 μm.

With respect to the distribution of $CNTF^+$ cells, there were no apparent differences between adult rat and mouse. In addition, there were no obvious differences in CNTF immunoreactivity or CNS distribution between adult C57BL/6J mouse and the mouse motoneuron mutant *mnd* (motoneuron degeneration 1),



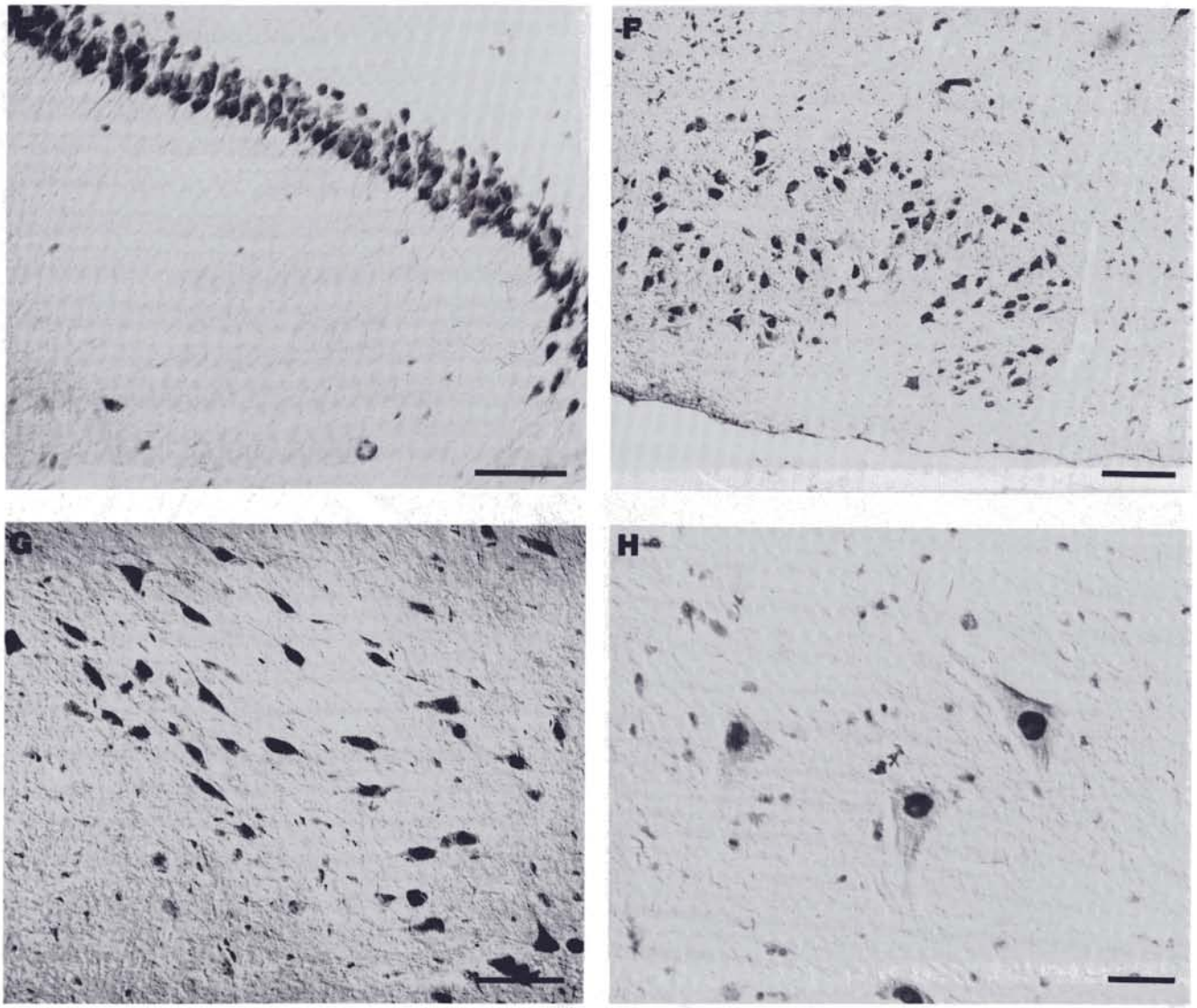


Fig. 5. Interference contrast photomicrographs of coronal sections through rat and mouse brain and spinal cord. A: CNTF immunoreactivity in the cortex of an adult *mnd / mnd* mouse. $CNTF^+$ neurons are found throughout all layers of the cortex, and there is a greater intensity of immunoreactivity in the nucleus as opposed to the cytoplasm of neurons. There does not appear to be any difference in subcellular localization in neurons in the mutant mouse as compared to rat or C57BL/6J mouse. Bar = 50 μm. B: $CNTF^+$ neurons in the adult C57BL/6J mouse olfactory bulb. Positive neurons appear in the glomerular layer as shown on the right hand side of the photomicrograph (g), as well as in bands of large positive neurons in the mitral cell layer (m). Bar = 100 μm. C: $CNTF^+$ neurons in the C57BL/6J mouse mesencephalon. Sections through the central grey area surrounding the cerebral aqueduct shows a number of $CNTF^+$ cells lining the aqueduct, as well as throughout the central grey region. A portion of the serotonergic medial dorsal raphe nucleus is shown, bounded by the two open arrows. Note that most neurons through the midbrain appear $CNTF^+$. Bar = 100 μm. D: $CNTF^+$ neurons in the rat hindbrain. The noradrenergic locus coeruleus nucleus is shown bounded by two open arrows. Note the $CNTF^+$ neurons throughout this portion of the hindbrain and the $CNTF^+$ Purkinje cells in the cerebellum (upper left). Bar = 100 μm. E: $CNTF^+$ CA1 neurons in the *mnd / mnd* mouse hippocampus. The majority of these neurons contain CNTF protein. Note the long processes which do not appear to contain any CNTF immunoreactivity. Bar = 50 μm. F: $CNTF^+$ cholinergic facial motoneurons from the *mnd / mnd* mouse. Note that these neurons do not appear to have decreased immunoreactivity in comparison to the rat or C57BL mouse sections displayed in this photoplate. Bar = 100 μm. (G) $CNTF^+$ rat dopaminergic neurons in the substantia nigra compacta. CNTF immunoreactivity is confined primarily to the cell soma in these neurons, with the nucleus displaying a greater degree of immunoreactivity as compared to the cytoplasm. Bar = 50 μm. H: $CNTF^+$ rat spinal motoneurons from the C_1, C_2 level of cord. Note that the cytoplasm of these neurons does not appear to be as intensely stained as neurons from rat brain, although there is still intense nuclear immunoreactivity. There appear to be many smaller glial cells which are immunoreactive for CNTF. Bar = 20 μm.

as shown in Fig. 5A,E,F. Analysis of early postnatal C57BL/6J mice (4 dpn) indicate sparse CNTF staining, with the majority localized to the glial limitans (data not shown).

$CNTF^+$ neurons were observed throughout the cerebral cortices. Fig. 5A shows a cortical section from an adult *mnd / mnd* mouse demonstrating positive neurons throughout all layers of the cortex. These neurons demonstrate a greater intensity of CNTF staining within the nucleus as opposed to the cytoplasm, as discussed below (Fig. 5G). Within the olfactory bulb, which has previously been shown to contain

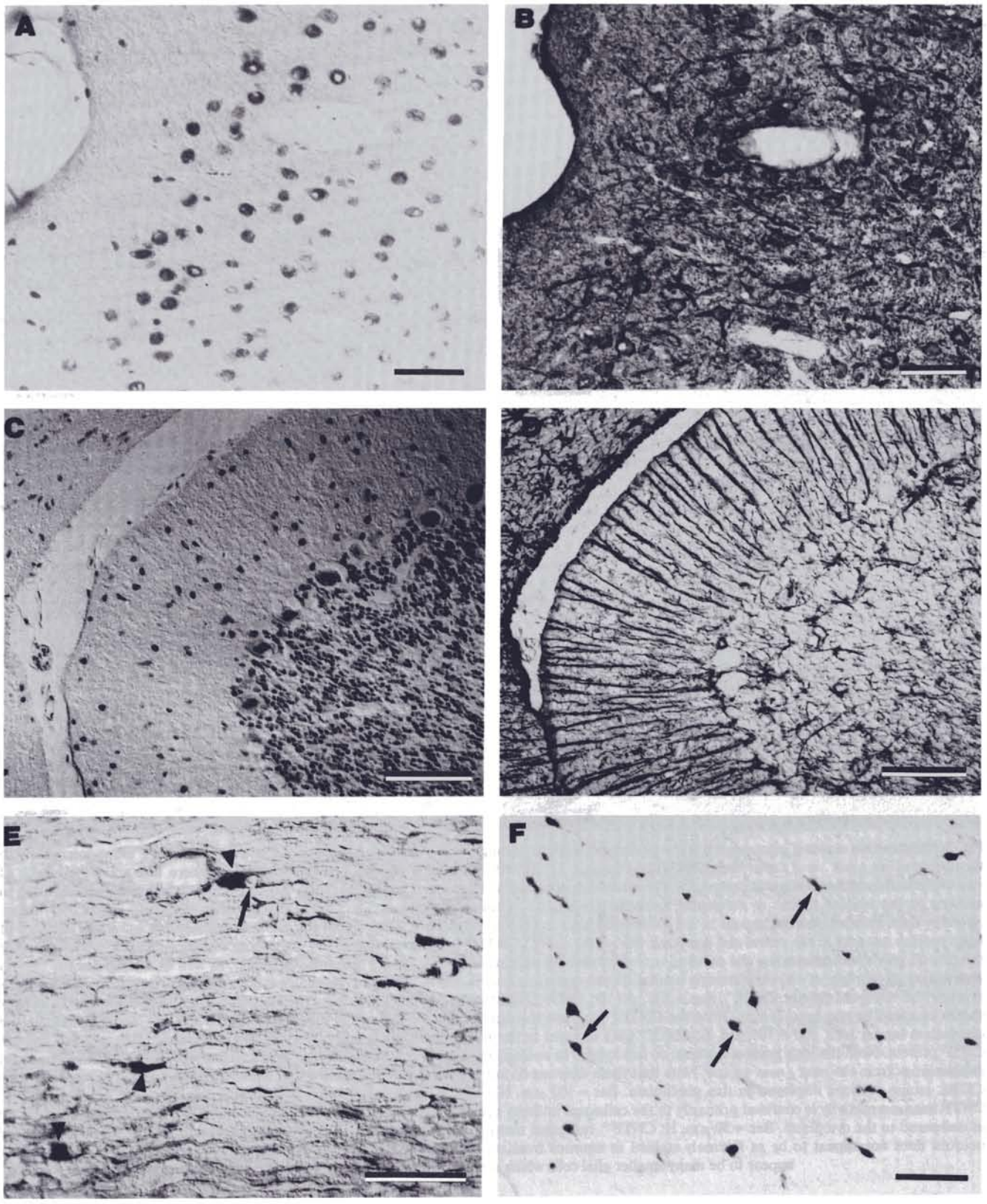


Fig. 6. Interference contrast micrographs through rat CNS and PNS illustrating CNTF glial and neuronal distribution. A,B: alternate serial sections reacted for CNTF (A) or phosphorylated neurofilament (B) through the cortex. Note the similar distribution of immunopositive cells indicating a neuronal localization of CNTF immunoreactivity. Bar = 50 μm. C,D: alternate serial sections reacted for CNTF (C) or GFAP (D) through the cerebellum. CNTF positive neurons are present both in the molecular layer and Purkinje cell layer. Positive cells in the granule cell layer are either glial or neuronal. Note that not all cells in the granular layer are $CNTF^+$. Bar = 100 μm. E: longitudinal section through sciatic nerve showing $CNTF^+$ Schwann cells. Arrowheads indicate strong immunoreactivity in the cytoplasm continuing out onto thin processes. Arrow indicates the Schwann cell nucleus with visible nucleolus which is not positive for CNTF. Bar = 50 μm. F: coronal section through the spinal tract of the mesencephalic trigeminal nerve showing $CNTF^+$ glial cells. Arrows indicate those cells which show some immunoreactivity in their processes. Here again, a greater degree of immunoreactivity appears in the region of the cell nucleus as opposed to the cytoplasm. Bar = 50 μm.

substantial amounts of CNTF activity[39], a large number of CNTF$^+$ neurons are observed throughout, for both rat and mouse tissues. Fig. 5B shows a section from a C57BL/6J mouse olfactory bulb illustrating positive neurons in both the glomerular and mitral cell layers.

CNTF$^+$ neurons were noted throughout the di- and mes-encephalon. There did not appear to be any specific association with a particular neurotransmitter system since the cholinergic, serotonergic, dopaminergic and noradrenergic neuronal populations could all be found to contain some degree of CNTF$^+$ neurons. Fig. 5C shows a section taken through the central grey area and the dorsal raphe, a serotonergic nucleus, immediately ventral to the cerebral aqueduct. Note the large number of intensely stained immunopositive ependymal cells surrounding the aqueduct, as well as the distributed nature of CNTF$^+$ cells in the central grey region. The strong CNTF staining of ependymal cells is interesting in view of the recent observation that the cerebrospinal fluid contains substantial quantities of CNTF α receptor under certain pathological conditions[5]. It is interesting to speculate that CNTF released from the natural turnover of these secretory cells could serve as source of functional CNTF/CNTFRα complexes which could in turn affect surrounding structures. Widespread CNTF staining is not however observed in the sub-ependymal region, which has previously been reported to contain significant levels of CNTF receptor α[18].

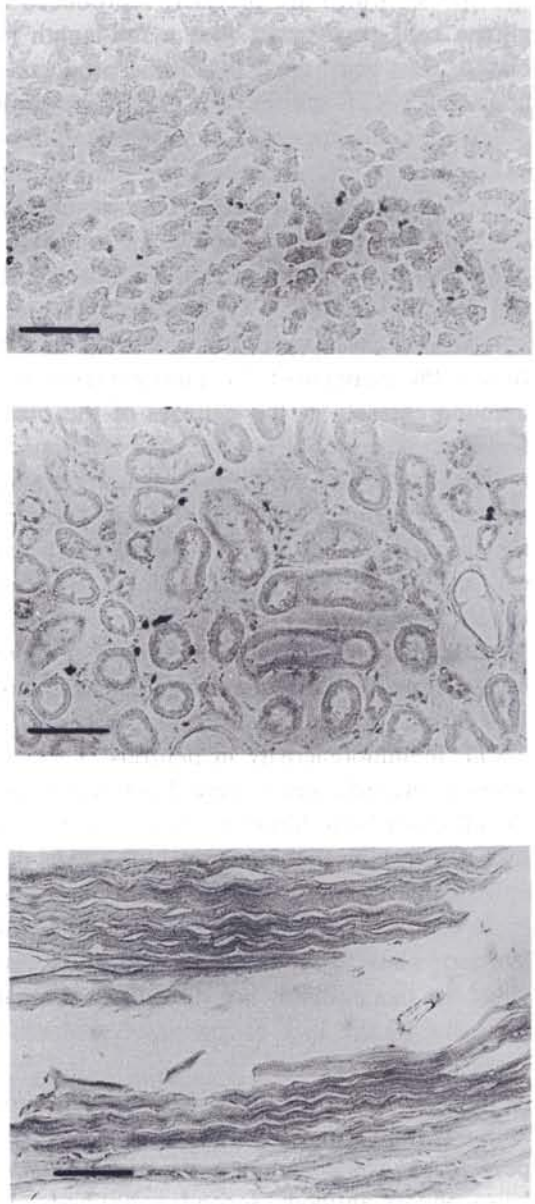


Fig. 7. Ten μm frozen sections stained for CNTF using HARC-1 antibody. Sections were treated as described in Materials and Methods. Sections of adult mouse show a similar pattern of reactivity (A) adult rat liver, (B) adult rat kidney, (C) adult rat muscle. Bars = 20 μm. No CNTF-specific staining is observed in these sections. In these sections, erythrocytes stain darkly due to their high content of alkaline phosphatase activity.

Fig. 5D illustrates CNTF$^+$ neurons in the locus coeruleus, a noradrenergic nucleus, as well as positive cells within the cerebellum and hindbrain. The CA1, 2, 3 and 4 neuronal populations of the hippocampus likewise were observed to contain CNTF$^+$ neurons, as did the dentate gyrus. Fig. 5E is taken through the CA1 area of an *mnd/mnd* mouse hippocampus showing CNTF localization primarily in the cell soma and not the processes. Cholinergic neurons of the facial motor nucleus were also positive for CNTF as shown in Fig. 5F. Substantia nigra compacta neurons, which are predominantly dopaminergic, were also CNTF$^+$ (Fig. 5G). We further examined the nuclear localization of CNTF within these neurons. The nuclei of these cells were optically 'sliced' in 1 μm steps using Metamorph imaging software in conjunction with a Ludl stage controller (provided by Dr. W.G. Tatton, Univ. of Toronto). Examination of a series of 5 slices through the cell revealed CNTF staining which extended throughout the nucleus but excluded the nucleolus, indicating the presence of CNTF within the cell nucleus. Sections taken through the rat spinal cord at the C_1–C_2 level demonstrate CNTF$^+$ cells throughout, including the spinal motoneurons as illustrated in Fig. 5H. Particularly within these spinal motoneurons, one can observe the pronounced nuclear distribution of CNTF, with lower amounts present within the cytoplasm. Note that the nucleolus of these cells do not exhibit immunoreactivity for CNTF. CNTF$^+$ neurons were also noted within the basal forebrain and the medulla.

Serial sections taken through the rat cortex were incubated with a monoclonal antibody to phosphory-

lated neurofilament proteins or with a monoclonal antibody to GFAP on alternate sections to those reacted for CNTF. Fig. 6A and 6B illustrate alternate sections taken through the rat cortex reacted for CNTF (Fig. 6A) or neurofilament protein (Fig. 6B), confirming that many of the observed $CNTF^+$ cells are indeed neurons. $CNTF^+$ cells were also noted throughout the granular and molecular layers of the cerebellum and within the Purkinje cell layer (see Fig. 6C). It is important to note however, that for both the molecular and granular layers, we observed a number of neighboring cells which were CNTF negative.

$CNTF^+$ glial cells were observed throughout the CNS although not with the same frequency as the immunopositive neurons, suggesting that perhaps only a subset of glia are immunopositive. A series of alternate sections through the cerebellum stained for GFAP (Fig. 6D) indicated that some of the $CNTF^+$ cells observed on the previous section (Fig. 6C) were indeed glial cells. However, $CNTF^+$ glial cells were more readily observed in coronal sections through the optic nerve, extrapyramidal tracts and cranial nerve tracts. In primary rat astrocyte cultures incubated with CNTF antibody, the pattern of increased nuclear immunoreactivity as compared to cytoplasmic was again observed (data not shown).

It has previously been reported that only myelinating Schwann cells express high levels of CNTF[10]. Using our anti-CNTF peptide antibody we found that many of the Schwann cells in the sciatic nerve are $CNTF^+$ and contain large amounts of CNTF protein (Figs. 6E and 3A). CNTF appeared to be primarily in the Schwann cell processes and soma, and interestingly *not* in the nucleus, in contrast to the CNTF sub-localization observed in neurons. With regard to CNS glia, CNTF staining was detectable in the glial cell body, as well as in occasional glial cell processes as shown in Fig. 6F. We observed a qualitatively similar pattern of CNTF distribution to that described above for Schwann cells, neurons and glia, using polyclonal antibodies which have been raised to the entire CNTF protein (provided by Dr. Peter Richardson).

DISCUSSION

This is the first report, to our knowledge, of the discrete localization of CNTF immunoreactivity to neurons in the CNS. We did not find evidence of a specific association between CNTF and a particular neurotransmitter system. In fact, CNTF may be best described as having an almost ubiquitous distribution among neurons in the normal rodent brain. We do however observe a general correlation between the level of CNTF staining in a given region and the level of CNTF bioactivity and mRNA described previously by Stockli and colleagues[39]. We also observe that CNTF may have a preferential disposition to the nucleus rather than the cytoplasm of neurons in the unperturbed adult rodent CNS. With regard to glia, we observe CNTF immunoreactivity in a sub-population of $GFAP^+$ astrocytes within the CNS, in cultures of purified 'type 1' cortical astrocytes, as well as myelinating Schwann cells of the sciatic nerve; consistent with previous reports[8,10,21,29,39]. Western analysis of these tissues indicates the presence of several immunoreactive species which are precisely reproduced by rat C6 glioma cells transfected with a full-length rat CNTF cDNA; thus confirming that these products represent bona fide CNTF species. The CNTF immunoreactivity observed in glia appear to be primarily in the cell soma as opposed to cell processes. We did not observe any noticeable diminution of CNTF immunoreactivity in the *mnd/mnd* mutant mouse, an animal which displays progressive motor deficits reminiscent of the clinical symptoms observed for amyotrophic lateral sclerosis (ALS), as compared to the normal C57BL mouse. In fact, there appears to be a modest increase in CNTF immunoreactivity in the neurons of the *mnd* mutant; however, since C57BL and *mnd* sections were not reacted under exactly identical conditions, we are unable to determine this conclusively at present. Outside the nervous system, no immunoreactivity is observed with affinity-purified HARC-1, for a variety of tissues tested.

Although our CNTF anti-peptide polyclonal antibody was made to a sequence similar to that of Peptide 1 of Stockli and colleagues[39], they did not report any CNTF immunoreactivity in neurons. Their survey examined the optic nerve, type 1 astrocyte cultures and the olfactory bulb; however, they did not observe positive neurons in the olfactory bulb. This discrepancy may be due to a difference in methods, antibody affinity or antigen specificity for the antibodies raised. The Western blots of sciatic nerve and brain extract provided for this antibody are difficult to compare to our data due to the lack of any size standards on these blots.

In order to insure the specificity of the anti-CNTF antibody–antigen complex, a number of controls were performed. These included pre-immune sera followed by secondary antibody, secondary antibody alone, antibody diluent followed by secondary antibody and primary antibody pre-blocked with either CNTF peptide or CNTF protein followed by secondary antibody. In each case, no specific pattern of immunoreactivity was observed. Specific staining was only observed in the

presence of either crude or affinity purified anti-CNTF peptide antibody, followed by secondary antibody. We have observed the described spatial pattern of staining both for our anti-peptide antibody, as well as with antisera raised to the entire CNTF protein. In addition, it should be noted that our tissue sections were kept on ice after cryosectioning until dry, and then stored at −20°C until needed. Past experience has shown that with some antisera, good immunoreactivity is lost when sections are air-dried at room temperature (N. Seniuk, unpublished data). We have found that the fixation (paraformaldehyde/glutaraldehyde for the mouse and paraformaldehyde for the rat) and extra blocking steps (0.1 M glycine as well as 10% normal serum) were critical to the success of a strong signal and low background in sections throughout the brain; while sections through optic nerve or sciatic nerve did not require the same blocking steps to achieve a good signal to noise ratio. In our attempts to co-localize GFAP or neurofilament (NF) immunoreactivity with CNTF, we found that the conditions required for good GFAP or NF signals reduced CNTF immunoreactivity, and we therefore chose to return to alternate sections in order to positively identify neurons and glia. Rende and coworkers[29] have raised polyclonal antisera to a 20 amino acid peptide from the C-terminus of rat CNTF (181–200) but apparently did not examine sections through the brain apart from the optic nerve.

Western analysis of the CNTF species present within the cerebral cortex using HARC-1 indicates a species of similar molecular weight to those observed from glial sources such as sciatic nerve and type 1 astrocyte cultures. With respect to the 45 kDa band observed in glial cell extracts, we believe that this species represents a modified form of CNTF rather than an immunologically related protein, since species of identical molecular weight are observed in extracts of sciatic nerve, CNTF transfected C6 glioma cells and recombinant CNTF (see Fig. 3, lanes 3, 4 and 6). As described in the results, this species has been observed previously[10,29], and is not observed in the parental C6 line. As with the other CNTF isoforms, we observed this species only within extracts of nervous tissue. The 45 kDa species is recognized by antibodies directed to several anti-CNTF epitopes as we observed it both with our affinity purified HARC-1 antibody and with polyclonal anti-CNTF antisera (PS 8–4) in the presence of a 100-fold excess of CNTF peptide. These conditions eliminate recognition of CNTF by HARC-1, indicating that recognition by PS 8–4 under these conditions is via an epitiope(s) distinct from those of HARC-1.

As to the origin of the 45 kDa species, several possibilities exist: (a) This may represent a form of CNTF dimer, a portion of which survives boiling in the SDS/β-mercaptoethanol containing loading buffer (or more probably represents reassociation during subsequent electrophoresis); or (b) this species represents a post-translational modification of CNTF, or (c) a combination of these two factors influence the formation of this species. We favor the latter hypothesis. It is known that while treatment with SDS largely disrupts protein tertiary structure, elements of secondary structure may be retained due to the negative change in free energy which these structures impart. Examples of this are enzymes which are functional at SDS concentrations equal to or above 1%, subsequent to boiling at 100°C (e.g., P1 nuclease, proteinase K, RNase A, to name a few). It should be noted that biologically active CNTF can be recovered following denaturation in agents such as urea, or from nitrocellulose filters following electrophoretic transfer from SDS–polyacrylamide gels[12]; indicating that CNTF can re-acquire a biologically active conformation following denaturation. It is possible that some portion of the CNTF in the extracts have re-acquired aspects of their secondary structure and associated to form the 45 kDa (dimer?) species. One possible site for this interaction are the N terminal 45 amino acid residues, which exhibit aspects of a leucine zipper. Self association of this complex might be stabilized by the interaction of cysteine residues which are present in this region *following* the re-association within the gel matrix (which lacks β-mercaptoethanol). Alternatively, cell-specific post-translational modifications of CNTF might further favor or retard this process. These possibilities are currently being examined in our laboratory.

At present we do not know whether the immunoreactivity observed in neurons represents a low level of endogenous transcription of CNTF, or is the result of uptake from non-neural cells. However, preliminary data from in situ hybridization studies in our laboratory indicate that both neurons and glia contain CNTF mRNA[37]. In this regard, it is interesting to note that the expression pattern of a CNTF promoter/β-galactosidase reporter construct has been reported in lines of transgenic mice[1]. Within these mice, specific Lac-Z expression is observed within Purkinje cells, hippocampal neurons and spinal motoneurons, as well as within the olfactory bulb, ciliary and facial ganglia, and dopaminergic neurons of the mesencephalon. These expression patterns may be representative of the endogenous pattern of CNTF expression within these cells.

The localization of $CNTF^+$ cells is perhaps not surprising when one considers the extensive functional similarities between CNTF and another motoneuron

trophic agent, brain-derived neurotrophic factor (BDNF). While the trophic actions of these factors are mediated by distinct receptor systems, these factors demonstrate an almost identical pattern of trophic abilities. Both proteins have been shown to promote the survival of spinal motoneurons[19,25], retinal ganglion neurons[20,41], cholinergic neurons of the basal forebrain and medial septum[14,32], dopaminergic neurons of the mesencephalon[15,41], as well as sensory and sympathetic neurons[31,32] at similar concentrations. In addition, both factors have been shown to rescue axotomized facial motoneurons and to prevent the developmental death of spinal motoneurons, to a strikingly similar extent[25,26,33,34,42]. These factors also share a number of neurite extension properties, and the ligand binding component of both receptors (CNTFRα and TrkB) are widely expressed within the CNS[18,32]. The signal transduction of both factors appears to involve tyrosine phosphorylation[17,40]. It is reasonable therefore that these factors might exhibit similar patterns of distribution within the CNS. BDNF has previously been localized to both glia and sensory, sympathetic and spinal motoneurons, as well as neurons of the hippocampus[19,32,43]. Yet although BDNF is present in populations such as spinal cord motoneurons, these neurons express the TrkB receptor and display enhanced survival in the presence of *exogenously* added BDNF[19,43]. A similar situation may exist for CNTF. Given the nuclear localization observed in CNTF$^+$ neurons, it will be interesting to see if a similar pattern of distribution exits for BDNF. Several other factors which have been shown to promote the survival of motoneurons and to exhibit some of the properties described above for CNTF and BDNF are the basic and acidic forms of fibroblast growth factor (FGF-1,2)[11,13]. Like CNTF these factors lack a canonical signal sequence and appear to exit the cell through unconventional mechanisms. These factors are also known to be present in both neurons and glia[2,28]. The neuronal and glial distribution of the trophic factors described above may therefore represent an important component of their actions on neural cells. In particular, the nuclear localization of CNTF immunoreactivity within a sub-population of neurons at a variety of CNS loci may be important in defining the functional parameters of these cells to exogenous agents. Given that functional CNTF receptors appear to be widespread on neurons of the adult rodent CNS, and that the primary expression of CNTF begins in the early postnatal period, it is possible that CNTF may provide a type of 'housekeeping' function within neurons following the establishment of stable synaptic contacts. This interaction could be either paracrine or autocrine in nature depending upon exact expression CNTF and its receptor in an individual neuron. The nuclear localization of CNTF also suggests the possibility that CNTF might interact with cellular components in a direct manner to affect gene transcription within neurons. We are currently examining these hypotheses.

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