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Ciliary neurotrophic factor prevents retrograde neuronal death in the adult central nervous system

(axotomy/cytokines/trophic factors)

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ABSTRACT **The neurocytokine ciliary neurotrophic factor (CNTF) was described originally as an activity that supports the survival of neurons of the chicken ciliary ganglia *in vitro*. The widespread expression of CNTF and its principal binding protein, CNTF receptor α, in the central and peripheral nervous systems suggests a broader trophic role for this peptide. In the present study, we report that CNTF prevents axotomy-induced cell death of neurons in the anteroventral and anterodorsal thalamic nuclei of the adult rat. Using the polymerase chain reaction, we also demonstrate the presence of CNTF and CNTF receptor α mRNA in these same thalamic nuclei. The coincidence of CNTF and its receptor in a population of neurons responding to the factor suggests a paracrine function for CNTF. The present findings establish that CNTF has significant effects on neurons of the central nervous system *in vivo* and demonstrate that neurocytokines can prevent cell death in the adult central nervous system.**

Neurocytokines [such as fibroblast growth factors, interleukins, transforming growth factors β, and ciliary neurotrophic factor (CNTF)] represent an emerging group of heterogenous pleiotropic peptides, many of which (e.g., interleukins) have been known previously for their effects on nonneural cells (1, 2). CNTF is increasingly recognized as the prototypical neurocytokine. *In vitro*, CNTF supports the survival of neurons of the peripheral sensory, sympathetic, and ciliary ganglia at various stages in their development (3–6) and induces the expression of choline acetyltransferase in sympathetic (7) and retinal (8) neurons as well as vasoactive intestinal peptide in embryonic sympathetic neurons (9). In culture, CNTF also causes the differentiation of the O-2A glial progenitor into a type 2 astrocyte in the developing optic nerve (10, 11). Immunocytochemical studies suggest that the expression of CNTF is localized to a subset of astrocytes in the central nervous system (CNS) (12) and Schwann cells in the peripheral nervous system (12, 13). Significantly, CNTF appears to be fundamentally different from members of the neurotrophin family [i.e., nerve growth factor (NGF), brain-derived neurotrophic factor, and neurotrophins 3, 4, and 5 (14–18)]. The CNTF gene lacks a signal peptide sequence, suggesting that CNTF may not be processed through classic secretory pathways (19, 20). On the basis of the previous feature, as well as the very late expression of the factor during development, it has been argued that CNTF is not a target-derived trophic factor (18). In contrast with NGF, CNTF or CNTF-like activity has not been demonstrated to be transported retrogradely (21). Whatever the mechanisms of action of CNTF, this factor protects avian motor neurons from developmental cell death (22) and prevents retrograde degeneration of axotomized motor neurons in the facial nucleus of the neonatal rat (23) and in preganglionic sympathetic neurons (6). CNTF also modifies retrograde changes of basal forebrain cholinergic neurons following axotomy (24). The previous studies indicate that CNTF promotes the survival of certain classes of neurons in the peripheral nervous system and may alter retrograde phenotypic changes in the CNS, an idea supported by the widespread expression of the principal binding protein for CNTF, CNTF receptor α (CNTFRα), in many areas of the nervous system (25, 26).

In the CNS, axotomy-induced degeneration of thalamic neurons represents the most classical example of retrograde cell death of central neurons (27). After axotomy, thalamic neurons undergo rapid retrograde degeneration, which becomes evident in the first week postlesion; this phenomenon has been largely exploited in early studies of thalamocortical connectivity (28–33). We have demonstrated previously that segments of peripheral nerve grafted into thalamus prevent, in part, axotomy-induced retrograde degeneration of these neurons and, in some instances, cause significant hypertrophy of axotomized cells (34). Because peripheral nerve segments are enriched in several neurotrophic factors, including CNTF (35), we hypothesize that CNTF might act as a neurotrophic factor in this setting. We report here that CNTF prevents axotomy-induced death of anterior thalamic neurons.

MATERIALS AND METHODS

Surgery. Adult male Sprague–Dawley rats were subjected to a unilateral aspiration of the cingulum bundle under sterile conditions through a window in the overlying sensorimotor cortex. This procedure transects axons of thalamic neurons in the anterodorsal (AD) and anteroventral (AV) nuclei that project, via the cingulum bundle, to the posterior cingulate cortex (36); this projection is a key link in the Papez circuit (37). The corpus callosum and fimbria-fornix were also removed to allow placement of a stainless steel cannula attached to an Alzet miniosmotic pump near the pia covering the anterior thalamus (38, 39) (Fig. 1). In CNTF-treated animals (n = 3), pumps contained 100 μg of recombinant rat CNTF (500 μg/ml) in storage vehicle solution (200 mM NaCl/20 mM Tris·HCl/0.1 mM EDTA/1 mM dithiothreitol). Forty microliters of artificial cerebrospinal fluid (ACSF; 122.6 mM NaCl/26.2 mM $NaHCO_3$/5.4 mM KCl/2.0 mM $MgSO_4$/1.2 mM NaH_2PO_4/2.0 mM $CaCl_2$/10 mM glucose) containing 0.1% rat albumin was added to the storage vehicle solution to obtain a final volume of 240 μl. In vehicle-treated rats, pumps contained either 200 μl of CNTF storage vehicle solution, to which 40 μl of ACSF with 0.1% rat albumin was added (n = 3), or 240 μl of ACSF with albumin alone (n = 1).

Histological Processing and Morphometry. Two weeks after surgery, animals were perfused via the aorta with ice-cold 0.1

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Abbreviations: CNTF, ciliary neurotrophic factor; CNS, central nervous system; NGF, nerve growth factor; AD, anterodorsal; AV, anteroventral; CNTFRα, CNTF receptor α.

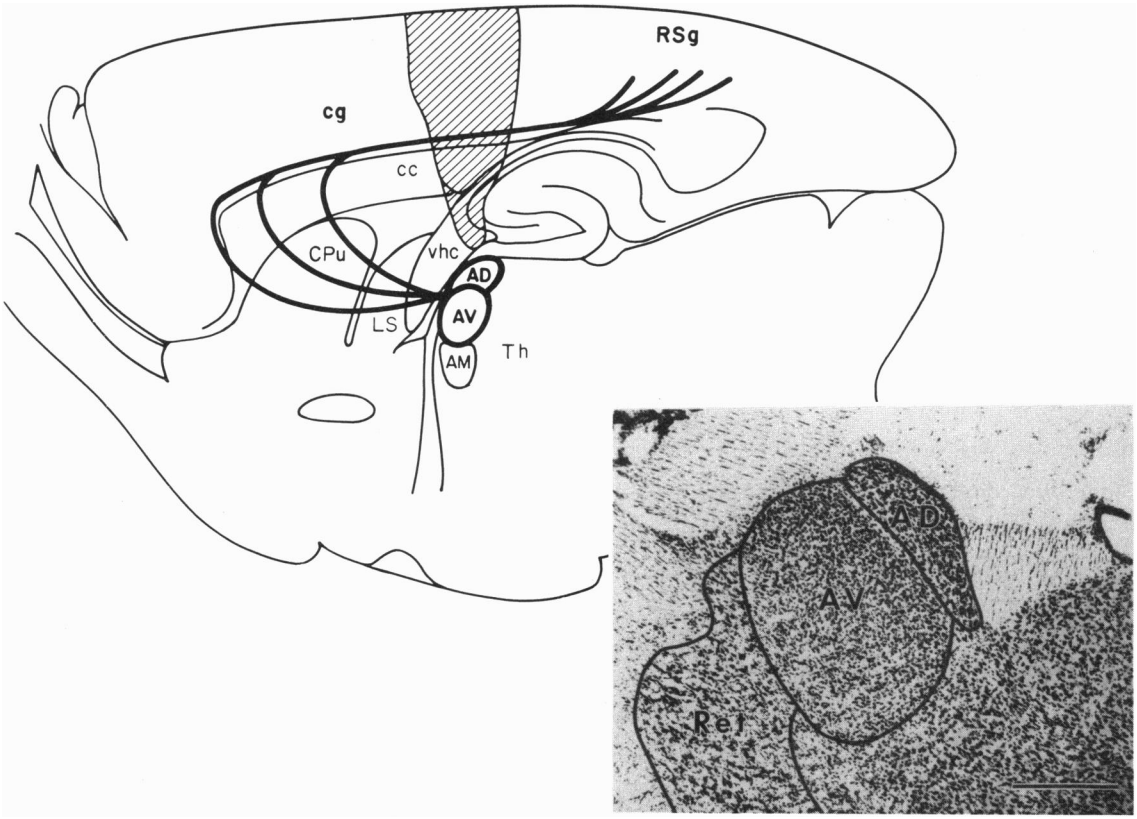

FIG. 1. Sagittal view of the rat brain showing the location of the anterior complex of the thalamus and the course taken by anterior thalamic axons en route to target fields in the retrosplenial granular cortex. Hatching represents tissue removed by the lesion. (*Inset*) Normal cytoarchitecture of the AV and AD nuclei in a Nissl-stained coronal section. AD, anterodorsal thalamus; AM, anteromedial thalamus; AV, anteroventral thalamus; cc, corpus callosum; cg, cingulate cortex; CPu, caudate-putamen; LS, lateral septum; Ret, reticularis; RSg, retrosplenial granular cortex; Th, thalamus; vhc, ventral hippocampal commissure. (Bar = 500 μm.)

M phosphate-buffered saline (1- to 2-min flush), followed by 350–600 ml of 4% freshly depolymerized paraformaldehyde. The brains were removed, dissected, blocked, and postfixed in 4% paraformaldehyde at 4°C for at least 48 hr. Animals were not included in the data set if delivery systems were found disconnected or obstructed. Blocks containing the anterior thalamus were dehydrated in graded alcohols and embedded in paraffin. Serial coronal sections (7 μm) were cut through the anterior thalamic complex, and every other section was stained with cresyl violet. Three sections per case (one through the middle of the anterior complex and two corresponding to planes at equal anterior and posterior distances from the middle level on each side of the brain) were taken for blind neuronal counts. Neurons were identified as Nissl-containing basophilic profiles with nucleoli and were counted at ×40 magnification, using a computerized image analysis system. A total of >15,000 cells were counted in six rats (three CNTF-treated animals and three vehicle-treated controls). Cell counts implemented in the study were aimed at providing a comparative estimate of the size of the anterior thalamic population (axotomized vs. control) and not an absolute number of surviving anterior thalamic neurons.

PCR, Cloning, and Sequencing. PCR was used to assay for the presence of CNTF mRNA in the anterior thalamus and its cortical target (retrosplenial cortex) or the presence of CNTFRα mRNA in anterior thalamus. Tissue cores were micropunched from these brain regions (40), and total RNA was isolated by homogenization in guanidinium and centrifugation through a cesium chloride cushion. Briefly, tissue cores containing AV and AD thalamic nuclei (≈0.5 mm in diameter, 0.3 mm thick) or retrosplenial cortex (≈1 mm in diameter, 0.3 mm thick) were placed in 150 μl of guanidinium homogenization buffer (4 M guanidinium thiocyanate/25 mm sodium citrate) and homogenized with a glass microhomogenizer. The homogenate was brought up to 1.5 ml with the same buffer and layered on top of 0.5 ml of cesium chloride (5.7 M cesium chloride/0.1 M EDTA). RNA was pelleted through the cushion by centrifugation for ≈18 hr at 41,000 rpm in a Sorvall RC M120 ultracentrifuge with a RP55S-212 rotor. The pellet was resuspended in 150 μl of diethyl pyrocarbonate-treated water and extracted with phenol/chloroform. Sodium acetate (15 μl of 3 M) was added, and the RNA was precipitated with 2.5 vol of isopropanol in the presence of glycogen. The RNA was pelleted in a microcentrifuge and washed with 70% (vol/vol) ethanol. The pellet was resuspended in 20 μl of diethyl pyrocarbonate-treated water and analyzed spectrophotometrically to determine yield and purity. To assay for CNTFRα mRNA, 1 μg of total RNA from anterior thalamus was used as a template for reverse transcription by Moloney murine leukemia virus reverse transcriptase primed by an antisense oligonucleotide (5′-CCGGAATTCCCAATCTCATTGTCCTTGGCTGC-CACCTGG-3′) complementary to bases 1097–1126 within the coding region of the human CNTFRα. This reaction mixture was then added to a PCR reaction with additional antisense primer as well as with a sense oligonucleotide (5′-CCGGTCGACCCACCATCAAGTACAAGGTCTCCAT-AAGTGTCAGC-3′) containing bases 781–815 within the coding region of the human CNTFRα gene and 281 bases upstream of the antisense primer (making the total length of the amplified product 364 bp). The PCR reaction was taken through 40 cycles. A similar procedure was used to assay for the presence of CNTF mRNA in retrosplenial cortex and anterior thalamus. The oligonucleotide primers used were 5′-CCGGAATTCGCGAATGGCTACATCTGCTTATCTT-TGGC-3′ (antisense) and 5′-CCGGTCGACGGATGGCTT-

TCGCAGAGCAAACACC-3′ (sense). To determine the size of amplified sequences, PCR products were electrophoresed through 2% agarose. To rule out genomic DNA contamination of the starting material, ≈200 ng of RNA from each sample was treated with DNase-free RNase prior to reverse transcription; such treatment resulted in no products. Final confirmation of the identity of amplified products was accomplished by cloning and sequencing of the amplified product for the CNTFRα and by restriction digestion with *Sty* I, *Hpa* I, and *Dde* I for CNTF. The 364-bp CNTFRα PCR product was blunt-end ligated into the *Sma* I site of a Bluescript KS II(+) plasmid vector and sequenced using Sequenase version 2.0. The sequence generated was analyzed using the MACVECTOR sequence analysis program (IBI/Kodak).

RESULTS

The cingulotomy procedures employed in the present study generated a simple and reproducible model of retrograde degeneration of rat AV and AD thalamic nuclei. Vehicle-treated animals showed profound retrograde degeneration in AV and AD nuclei on the lesioned side, whereas CNTF-treated animals exhibited a significant reduction in the magnitude of retrograde degeneration (Fig. 2). In the AV nucleus of vehicle-treated animals (n = 3), there was ≈75% reduction in the number of cells; additional evidence of retrograde degeneration was also present, including marked gliosis and thalamic retrograde dust, a particulate deposit characteristic of degenerating thalamic neurons (42) (Fig. 2, compare *A* and *C*; Fig. 3). The magnitude of cell loss was decreased to 34% in CNTF-treated animals (n = 3), and other evidence of degeneration was either ameliorated (gliosis) or abolished (retrograde dust) (Fig. 2, compare *C* and *E*). Residual cells appeared somewhat less basophilic than anterior thalamic neurons on the contralateral side. In the AD nucleus, there was a 56% reduction in the number of cells in untreated lesioned animals but only a 20% reduction in CNTF-treated rats (Fig. 2 *B*, *D*, and *F* and Fig. 3).

To examine the possible physiological relevance of CNTF for thalamic neurons, we assayed for the presence of CNTF mRNA in the anterior thalamus and retrosplenial cortex and for the presence of CNTFRα mRNA in the anterior thalamus using PCR. Oligonucleotide primers were designed based on the published sequences for rat CNTF (20) and human CNTFRα (25). This approach yielded amplified products that migrated at the expected sizes of 364 bp in the case of CNTFRα, or ≈631 bp in the case of CNTF (Fig. 4 *A* and *B*). The CNTFRα product was subsequently cloned and sequenced to confirm that it represents the rat homologue of the human CNTFRα. The cloned fragment contained a portion of the rat CNTFRα, demonstrating 87% nucleotide sequence identity and 99% identity in the deduced amino acid sequence with the human receptor (Fig. 4*C*). The identity of the CNTF PCR product was confirmed by restriction enzyme digestion.

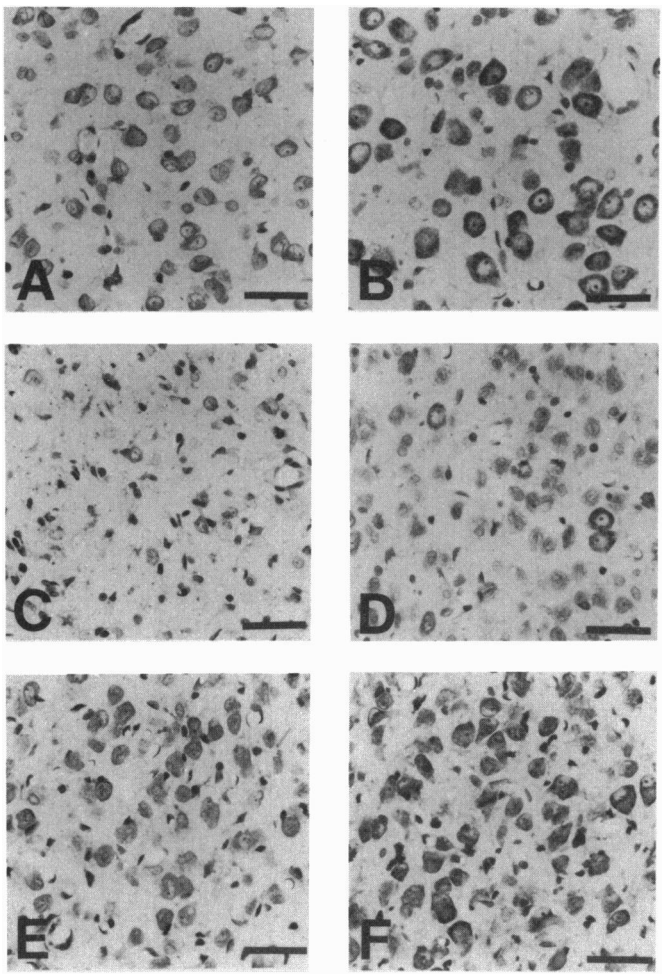

FIG. 2. CNTF prevents retrograde degeneration of AV and AD thalamic neurons. (*A* and *B*) Nissl-stained sections (7 μm) through the anterior thalamus, contralateral to the lesion, show numerous healthy basophilic neurons in AV (*A*) and AD (*B*) nuclei. (*C* and *D*) On the lesioned side of vehicle-treated animals, there is profound retrograde degeneration of these cells, including reduced basophilia, cell atrophy, and cell loss. Note the marked gliosis in these nuclei, presumably consisting of proliferating microglia (41) and also the retrograde thalamic dust (punctate material in upper left quadrant of *C*), not as apparent in these black-and-white figures because of its metachromatic (pink) reaction to cresyl violet. (*C*) AV neurons. (*D*) AD neurons. (*E* and *F*) Retrograde changes in neurons in *C* and *D* are ameliorated by treatment with CNTF. (Bars = 50 μm.)

DISCUSSION

The present study demonstrates that CNTF prevents axotomy-induced retrograde death of neurons in AV and AD thalamic nuclei. This finding is the second major protective effect of a trophic factor shown in the CNS, after the demonstration of the effects of NGF on injured basal forebrain cholinergic neurons (43). More importantly, this observation provides evidence that trophic factors can prevent

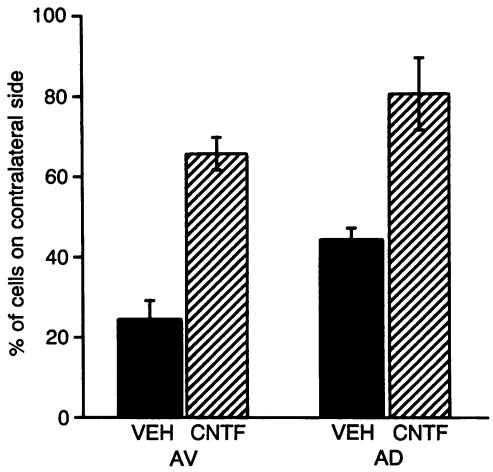

FIG. 3. Histogram of the number of axotomized AV and AD neurons in vehicle (VEH)-treated (n = 3) and CNTF-treated (n = 3) animals. The neuron number is expressed as a percentage of cells counted on the contralateral (unlesioned) side. Differences are statistically significant by Student's t test (AV, P = 0.003; AD, P = 0.018).

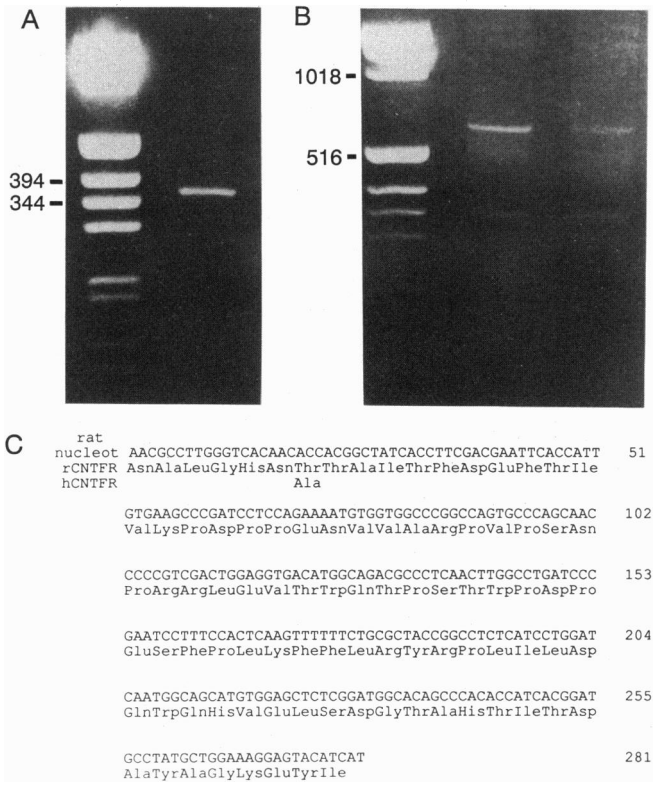

FIG. 4. PCR analysis of CNTF and CNTFRα expression. (*A*) Ethidium-stained 2% agarose gel of PCR product generated with cDNA from anterior thalamic RNA and human CNTFRα oligonucleotide primers. The amplified fragment migrated at ≈364 bp, the size expected based on the human CNTFRα sequence. (*B*) PCR product generated with cDNA from anterior thalamic RNA (lane closest to size markers) or retrosplenial cortex RNA (lane farthest from size markers) and rat CNTF oligonucleotide primers. The amplified product migrated at ≈631 bp, the size expected based on the rat CNTF sequence. Size markers are a 1-kb DNA ladder (BRL). (*C*) Confirmation of the identity of the CNTFRα PCR product by cloning and subsequent sequence analysis. Only 1 of the 93 amino acids encoded by the amplified rat CNTFRα fragment is different between rat and human receptors.

neuronal death in the CNS. Although the effects of NGF in the lesioned septohippocampal pathway represent the best-characterized CNS model of trophic action *in vivo*, it is unclear whether, in that setting, NGF protects septal cholinergic neurons from retrograde degeneration or merely stimulates their phenotypic features. After complete lesions of the fimbria-fornix, cholinergic neurons of the medial septal nucleus die 3–4 weeks postaxotomy (44, 45, 50). However, using this type of lesion, NGF treatment has been limited to 2 weeks postaxotomy (i.e., a period of time in which retrograde cell death is negligible). Certainly, follow-up of cingulectomized, CNTF-treated animals over a range of survival times exceeding 2 weeks will be necessary to ensure that the rescue effect of CNTF is maintained.

Several studies have begun to examine the localization of CNTF in the nervous system and report widespread expression of both mRNA and protein (12, 13). Consistent with these findings, we have localized CNTF mRNA in the cortical targets and local environment of anterior thalamic neurons. This evidence, in conjunction with available information concerning the principal CNTF-binding protein CNTFRα (25, 26, 46), provides a basis for an understanding of the role for CNTF in the nervous system. Although the widespread expression of CNTF may not be compatible with a role of this peptide as a target-derived trophic factor, CNTF does appear to support the survival of neurons in the neonatal peripheral nervous system and adult CNS, as demonstrated by its effects on embryonic motor neurons during developmental cell death (22) and on axotomized neonatal motor neurons (23), as well as anterior thalamic neurons (present study).

It has been suggested recently that the effects of CNTF on target cells may require the presence of at least two receptors, one of which is CNTFRα, a binding protein selective for CNTF, and the other being gp130, a signal-transducing receptor shared among CNTF, interleukin 6, and the leukemia inhibitory factor (26). In the present study, we have documented the presence of both CNTF and CNTFRα mRNA in anterior thalamus. We have also localized gp130 mRNA in the same brain structure by using the same procedure as for CNTF and CNTFRα (R.E.C. and V.E.K., unpublished results). The concomitant expression of CNTF and its two main receptor proteins in a population of neurons responding to CNTF suggests a possible paracrine role for this peptide in the CNS. CNTF may be released locally from stimulated glial cells. Stimulated glia can express neurotrophins (e.g., NGF) (47), as well as other growth factors, and various cytokines have the ability to regulate this expression (48). CNTF itself might be capable of binding to glia directly and inducing the release of additional factors in an autocrine fashion. Although the direct demonstration of functional CNTF receptors has been, thus far, limited primarily to neurons (46), glial progenitor cells in optic nerve cultures do respond to CNTF (10, 11). The localization of CNTFRα and gp130 in distinct cell populations within AV and AD nuclei should indicate whether the protective effect of CNTF on thalamic neurons is a direct effect on these cells or is mediated through glial elements via complex cascades, as suggested for other cytokines (49). Further understanding of the CNTF signal transduction (26) as well as detailed knowledge of the cellular localization of the various receptors involved will be necessary to understand the potential interactions in the CNTF trophic cascades.

In the present report, the thalamic axotomy paradigm was used as a classical example of retrograde degeneration in the CNS and not as a model to demonstrate the selective effects of CNTF. The widespread expression of CNTF and its receptor suggests that this factor may promote the survival of broader populations of CNS neurons undergoing cell death in the course of neurodegenerative disorders or in other types of neurological injury (e.g., anoxia-ischemia injury).

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