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A role for ciliary neurotrophic factor as an inducer of reactive gliosis, the glial response to central nervous system injury

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ABSTRACT **Within the central nervous system (CNS) ciliary neurotrophic factor (CNTF) is expressed by astrocytes where it remains stored as an intracellular protein; its release and function as an extracellular ligand are thought to occur in the event of cellular injury. We find that overexpression of CNTF in transgenic mice recapitulates the glial response to CNS lesion, as does its injection into the uninjured brain. These results demonstrate that CNTF functions as an inducer of reactive gliosis, a condition associated with a number of neurological diseases of the CNS.**

Ciliary neurotrophic factor (CNTF) promotes the survival of a variety of neuronal populations in culture (1–4). It also acts as a differentiation and trophic factor on glial cells *in vitro* (5, 6). *In vivo*, exogenously added CNTF prevents the death of damaged neurons in several injury models (7, 8) and inhibits the onset of symptoms associated with progressive motor neuron degeneration in the *pmn* mutant mouse (9). The function of CNTF as a ligand is thought to be limited to instances that involve cellular injury because CNTF is a nonsecreted cytosolic protein that lacks a consensus secretory signal sequence (10–12). However, the physiological role of CNTF upon injury remains unknown.

Ablation of the murine *cntf* gene has revealed an essential role for CNTF in postdevelopmental motor neuron maintenance (13); CNTF-null mice do not begin to show an abnormal phenotype until adulthood, when they exhibit atrophy and loss of motor neurons and diminished muscle strength. Based on these results, it has been proposed that microtrauma caused by normal physical stress permits the release of CNTF into the extracellular environment where it exerts its trophic action in maintaining motor neuron viability.

CNTF has been shown to influence the differentiation of glial precursor cells *in vitro* (5, 6); however, evidence for an ontogenetic role in this process *in vivo* is lacking. By using transgenic mice that overexpress CNTF, we assessed the possible function of CNTF in the development of astrocytes and oligodendrocytes. We find that mice that overexpress CNTF display no overt developmental glial abnormalities. However, elevated levels of CNTF in transgenic mice lead to the induction of a glial phenotype, reactive gliosis, that is characteristically observed in cases of central nervous system (CNS) injury and disease (14, 15). Further, exogenous CNTF supplied by intracerebral injection also led to gliosis in normal mice. These results support the proposal that a function of CNTF in the CNS may be in regulating the glial response to neural lesion.

MATERIALS AND METHODS

Production of *cntf* Transgenic Mice. Transgenic mice were generated by using 5.3 kb of murine genomic DNA, including the *cntf* coding region and 2.3 kb of endogenous upstream sequence. The 2.3-kb upstream sequence spans the entire intergenic distance between *cntf* and the gene immediately preceding it, which is known as *pzf* (16). Therefore, it is likely that the transgene construct contains all of the endogenous CNTF promoter. The transgene was microinjected into fertilized eggs from CBA/C57BL mice. Eggs were subsequently reimplanted into pseudopregnant foster mothers by standard techniques (17). Genomic DNA from biopsies of the tails of the offspring were analyzed to identify mice carrying the *cntf* transgene. Three single-locus transgenic mouse lines were established, each with a single distinct site of transgene integration. The number of transgene copies harbored by each line was determined by densitometric scanning of genomic Southern blots. In all comparative studies, heterozygous transgenic mice were compared to their wild-type litter mates. Mice were analyzed between 8 and 16 weeks of age.

CNTF and Glial Fibrillary Acidic Protein (GFAP) Expression in Transgenic Tissue. The level of *cntf* transcription was measured by scanning densitometry of the 1.2-kb mRNA species on a Northern blot autoradiograph. *cntf* mRNA levels are reported relative to wild-type brain (excluding olfactory bulb and optic nerve) and normalized by using mRNA for glyceraldehyde phosphate dehydrogenase (GAPDH) as an internal standard. CNTF protein levels were determined in whole brain, including olfactory bulb, by using a chicken sympathetic chain neuronal survival assay (18). Recombinant human (rh) CNTF was used as a standard to calculate the amount of CNTF present in the mouse tissue extracts. The levels of mRNA for GFAP were determined by Northern blot analysis of total RNA isolated from the olfactory bulbs of wild-type or transgenic mice. A 540-bp *Sca* I–*Spe* I fragment of the rat *gfap* cDNA was used as a hybridization probe. Quantification of band intensity was performed with a Packard InstantImager. Values were corrected for differences in the amounts of total RNA loaded by using mRNA levels for GAPDH. *gfap* mRNA was measured in three samples of wild-type and transgenic olfactory bulb total RNA and found to be similar in all samples.

Immunohistochemistry and Carnosine Determination. Adult mice were perfused with 4% (wt/vol) paraformaldehyde; olfactory bulbs were removed, postfixed in 4% paraformaldehyde, incubated in 30% (wt/vol) sucrose, snap-frozen, and embedded for sectioning. Spinal cords were dissected from perfused animals after *in vivo* postfixation overnight at 4°C and then embedded in freezing medium. A cryostat microtome was used to cut 10-μm coronal sections of the olfactory bulbs and 20-μm longitudinal sections of the spinal cords. Sections were mounted on gelatin-coated glass microscope slides. Sections were treated with 0.3% hydrogen peroxide and incubated sequentially with phosphate-buffered saline (PBS), mouse anti-GFAP monoclonal antibody (Boehringer Mannheim) di-

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Abbreviations: CNS, central nervous system; CNTF, ciliary neurotrophic factor; GFAP, glial fibrillary acidic protein; OMP, olfactory marker protein; rh, recombinant human.

luted 1:50 in PBS containing 1% normal goat serum or rat anti-Mac-1 monoclonal antibody (Boehringer Mannheim) diluted 1:100, twice with 1% normal goat serum, and either biotin-labeled goat anti-mouse IgG $F(ab')_2$ fragment (for GFAP) or biotin-labeled goat anti-rat IgG $F(ab')_2$ fragment (for Mac-1). Olfactory marker protein (OMP) staining was carried out as described above, except that goat anti-OMP was used at a dilution of 1:5000 and a biotin-conjugated rabbit anti-goat $F(ab')_2$ fragment was used as a secondary antibody. An ABC peroxidase detection system (Vector Laboratories) was used for the final staining steps. Olfactory bulb carnosine levels were measured by a spectrophotometric method (19). Experimental values were extrapolated from a standard curve generated by using pure carnosine (Sigma).

Induction of Neuronal Degeneration. To induce sensory neuron degeneration, wild-type mice were treated intranasally with $ZnSO_4$ as described (20).

Stereotactic Injection of CNTF. CNTF protein (0.36 μg of rhCNTF) was administered intracerebrally by using a stereotactic injection apparatus. Heat-treated CNTF was used as a negative control. It was shown independently to have no biological activity in the chicken sympathetic chain neuronal survival assay (18). In the same assay, the native rhCNTF used in this experiment was shown to be fully active. SDS/PAGE and silver staining were performed on the supernatants of treated and untreated samples to determine that the protein remained in solution and had not been degraded or lost during the inactivation step. The photomicrographs shown in Fig. 3 are representative of the eight mice injected with active CNTF and the four injected with inactivated protein.

RESULTS

Generation of *cntf* Transgenic Mice. We produced several lines of transgenic mice by using 5.3 kb of genomic DNA including the CNTF coding region and 2.3 kb of endogenous upstream sequence in an effort to maintain the wild-type pattern of expression. Each transgenic line expressed CNTF at levels proportional to its gene copy number; one line (t30) harbored 30 copies of the *cntf* gene and expressed 27 times the level of CNTF found in wild-type whole brain; another line (t3) with three extra copies of the gene expressed three times the level of CNTF (Table 1).

CNTF Overproduction Results in Reactive Gliosis in the Olfactory Bulb of *cntf* Transgenic Mice. Astrocyte and oligodendrocyte populations in the CNS of wild-type and *cntf* transgenic mice (t30) were compared immunohistochemically in coronal sections of the entire brain. GFAP (21) and the π isoform of glutathione *S*-transferase (22) were used as markers for astrocytes and oligodendrocytes, respectively. Mice overexpressing CNTF contained many more $GFAP^+$ cells within the olfactory bulbs than did wild-type mice (Fig. 1 *A* and *B*), but no differences were found elsewhere in the brain. Oligodendrocyte staining did not differ between wild-type and transgenic brains (data not shown).

Table 1. Overexpression of CNTF in the CNS of transgenic mice

	CNTF			
		RNA		
Mouse	DNA	Brain	Olf. bulb	Protein
Wild type	2	1	2	17
cntf transgenic				
t3 line	5	ND	6	50
t30 line	30	10	40	442

DNA (*cntf* copy number) and transcription (relative levels of RNA in brain and olfactory bulb) and protein (ng of rhCNTF equivalents per mg of total protein) levels were estimated. ND, not determined.

GFAP staining in the wild-type olfactory bulb was relatively weak and largely confined to the glomerular region (Fig. 1*B*). In the transgenic mice, $GFAP^+$ cells were present throughout the olfactory bulb with densely stained plaques of GFAP found in the glomeruli (Fig. 1*A*). Astrocytes of the transgenic olfactory bulb were hypertrophied and had thick intensely staining $GFAP^+$ processes (Fig. 1*D*) characteristic of reactive astrocytes associated with insult and disease in the CNS (14, 15). This contrasts with the appearance of the astrocytes in wild-type mice (Fig. 1*E*). Elevation in GFAP staining was also observed in the olfactory bulb of another CNTF transgenic line (t3), indicating that this phenotype is independent of the locus of transgene integration.

In the CNS, the Mac-1 antigen (CD11b) is expressed by brain macrophages and microglia (23). These phagocytic scavenger cells are a prominent feature of reactive gliosis in areas of CNS lesion (24). Staining with an antibody against Mac-1 revealed the presence of microglia/brain macrophages in the olfactory bulbs of the *cntf* transgenic mice, specifically within the glomerular layer (Fig. 1*F*). The olfactory bulbs of wild-type mice were devoid of $Mac\text{-}1^+$ cells (Fig. 1*G*). In summary, the bulbs of transgenic mice overexpressing CNTF exhibit three salient characteristics of reactive gliosis: induction of GFAP, hypertrophy of astrocytes, and the presence of microglia (15, 24).

Chemical insult to the olfactory epithelium is known to cause sensory neuron degeneration and reactive gliosis in the olfactory bulb (20). The phenotype of the transgenic olfactory bulb was strikingly similar to the gliotic condition seen in the bulbs of mice with chemically induced sensory neuron degeneration caused by intranasal exposure to $ZnSO_4$ (Fig. 1*C*). Because of this similarity and the fact that gliosis generally occurs in conjunction with neuronal insult (15), we analyzed the olfactory bulbs of the transgenic mice for signs of neuronal abnormalities. Innervation of the bulb by sensory neurons was indistinguishable in transgenic and wild-type mice, as judged by immunohistochemical staining for OMP (25) (Fig. 2 *A* and *B*). Olfactory bulb OMP immunoreactivity diminished significantly when the afferent fibers degenerated after chemical insult (Fig. 2*C*). Another marker for olfactory sensory neurons, the dipeptide carnosine (26), was also unchanged in transgenic mice (1.12 ± 0.08 and 1.21 ± 0.14 nmol/mg of protein for wild-type and transgenic mice, respectively). In contrast, mice treated with $ZnSO_4$ had reduced levels of bulbar carnosine (0.45 ± 0.01 nmol/mg of protein). In addition, no detectable olfactory deficit was found in the mice that overexpressed CNTF (data not shown); the transgenic mice bred and nursed normally and were able to locate hidden food, a task anosmic mice are incapable of performing (27).

These experiments suggest that the gliotic condition found in the olfactory bulb of the *cntf* transgenics occurs in the absence of neuronal abnormalities. Also, reactive gliosis was notably absent from other regions of the transgenic brain as examined by GFAP staining. The specific effect of CNTF overexpression in the bulb may result from normal neuronal turnover that is unique to the olfactory system (28). The cell death occurring as part of turnover may allow the release of CNTF from intracellular storage in the glial cells. Consistent with this idea, CNTF is synthesized by glial cells associated with these same short-lived neurons in the bulb (29). Perhaps the gliotic effect of CNTF overexpression is not evident in other areas of the CNS because the ligand remains sequestered intracellularly under basal physiological conditions.

***gfap* mRNA Levels.** The level of *gfap* mRNA in the olfactory bulb of *cntf* transgenic mice is elevated 2-fold over wild-type levels, as determined by Northern blot analysis and quantification of band intensity. It seems likely that the increased GFAP immunoreactivity is due at least in part to increased GFAP expression. This result contrasts with the observation that GFAP

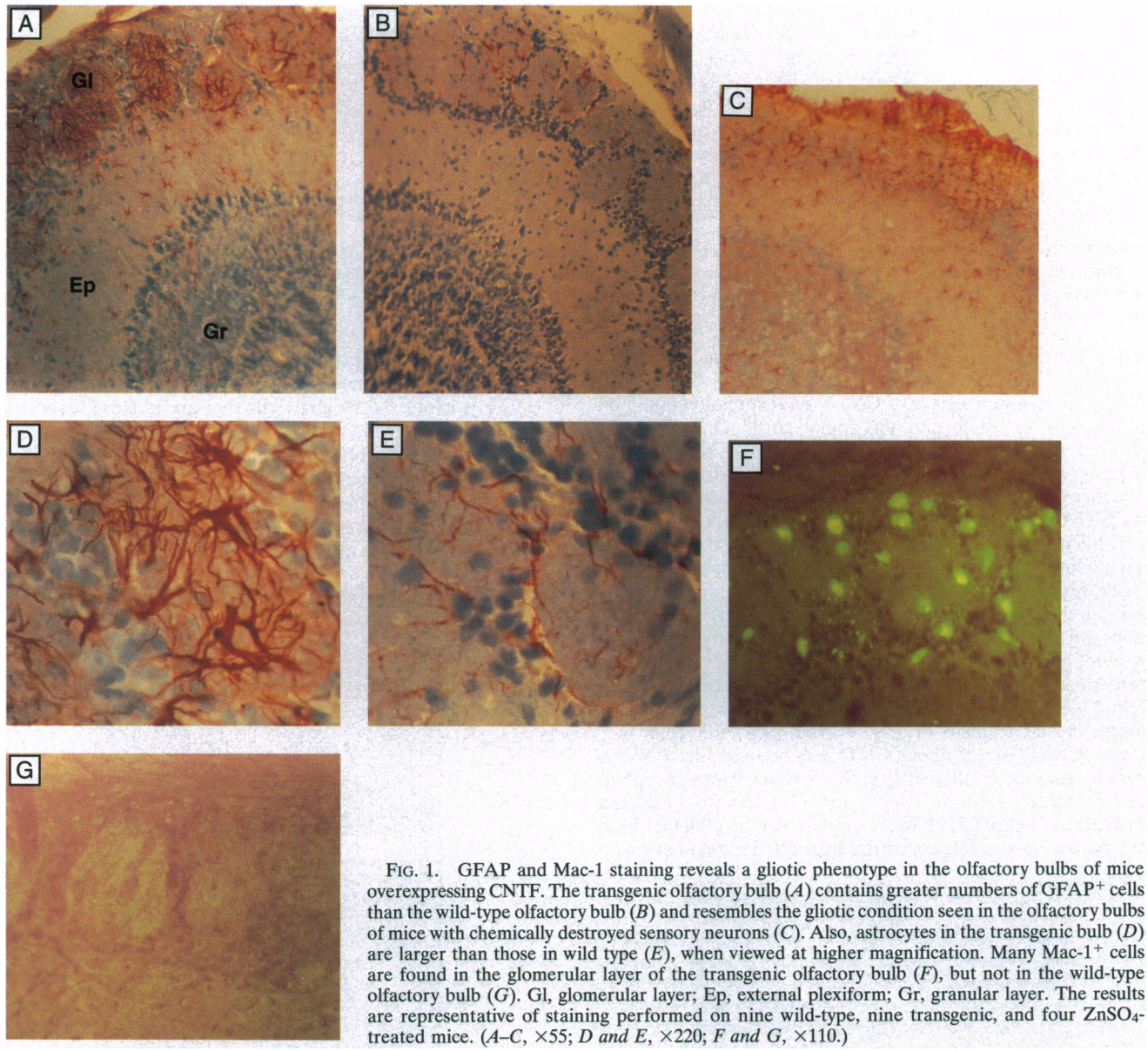

FIG. 1. GFAP and Mac-1 staining reveals a gliotic phenotype in the olfactory bulbs of mice overexpressing CNTF. The transgenic olfactory bulb (*A*) contains greater numbers of GFAP$^+$ cells than the wild-type olfactory bulb (*B*) and resembles the gliotic condition seen in the olfactory bulbs of mice with chemically destroyed sensory neurons (*C*). Also, astrocytes in the transgenic bulb (*D*) are larger than those in wild type (*E*), when viewed at higher magnification. Many Mac-1$^+$ cells are found in the glomerular layer of the transgenic olfactory bulb (*F*), but not in the wild-type olfactory bulb (*G*). Gl, glomerular layer; Ep, external plexiform; Gr, granular layer. The results are representative of staining performed on nine wild-type, nine transgenic, and four $ZnSO_4$-treated mice. (*A–C*, ×55; *D and E*, ×220; *F and G*, ×110.)

immunoreactivity alone increases in the reactive gliosis associated with experimental allergic encephalomyelitis (30).

CNTF Causes a Widespread Gliotic Response When Stereotactically Injected into the Brain of Wild-Type Mice. If the specificity of CNTF-induced gliosis in the transgenic is due to its exceptional release in the bulb, delivery of extracellular CNTF should be sufficient to elicit gliosis in other areas of the brain. To test this possibility, we analyzed the effect of CNTF on GFAP expression after intracerebral administration. Wild-type mice were stereotactically injected with 0.36 μg of rh-CNTF. After 48 h, the mice were immunohistochemically analyzed for GFAP expression. CNTF injection resulted in a widespread increase in the number of GFAP$^+$ cells (Fig. 3*A*). The induction of GFAP was apparent in many regions of the CNTF-injected hemisphere, relative to the uninjected contralateral side. A dose–response experiment indicated that as little as 50 ng of CNTF was capable of eliciting a widespread GFAP induction. In contrast, mice injected with an equal dose of heat-inactivated rhCNTF did not exhibit a change in GFAP staining except at the site of the wound created by the injection needle (Fig. 3*B*). These results demonstrate that CNTF has widespread actions as an inducer of reactive astrocytes. We observed no change in Mac-1 staining in the CNTF-injected mice, suggesting that a microglial response is not essential for the production of reactive astrocytes.

Gliosis in the Spinal Cord of *cntf* Transgenic Mice. The results of the *cntf* gene knockout study (13) prompted us to analyze the spinal cord of the mice overexpressing CNTF for evidence of gliosis. We found an increase in the level of astrocytic GFAP staining in the transgenic spinal cord relative to wild-type littermates (Fig. 4 *A* and *B*). As was observed in the olfactory bulb, the astrocytes in the transgenic spinal cord also appeared hypertrophied when compared to the astrocytes of the wild-type spinal cord. A number of these astrocytes appear to be wrapped around neuronal cell bodies (Fig. 4*C*). No elevation in Mac-1 staining for microglia was seen in the transgenic spinal cord in contrast to the transgenic olfactory bulb.

DISCUSSION

These results indicate that the function of CNTF in the CNS is not limited to its trophic activity on neurons but also includes effects on glia as a signal for reactive gliosis. A role for CNTF in the induction of reactive gliosis is consistent with the fact that the release of CNTF from the cell and the development

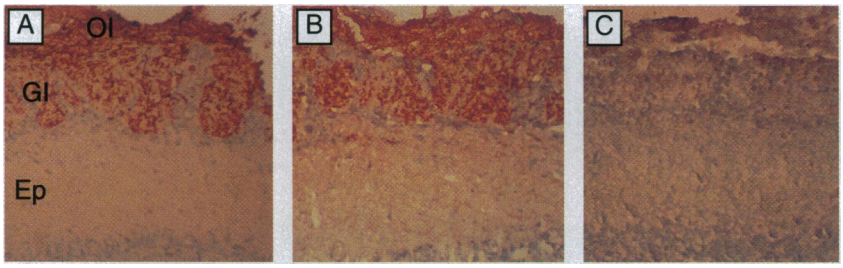

FIG. 2. Transgenic olfactory bulb appears normally innervated by primary olfactory sensory neurons. The levels of OMP, a marker for mature olfactory receptor neurons, in the olfactory bulb of transgenic mice (*A*) and wild-type mice (*B*) are indistinguishable. The $ZnSO_4$-treated mice (*C*) show very little OMP staining as a result of degeneration of the axons of olfactory receptor neurons. Ol, olfactory nerve layer; Gl, glomerular layer; Ep, external plexiform. (×50.)

of the gliotic condition both occur in response to cellular damage.

Why is the gliotic effect of CNTF overexpression common to the olfactory bulb and the spinal cord? One similarity between these two areas of the CNS is that both contain neurons with projections to the periphery. Also, both the olfactory bulb and the spinal cord express relatively high levels of CNTF in rodents (31). One possibility is that the spinal cord and olfactory bulb provide a mechanism to release CNTF from cytoplasmic storage—namely, through physical stress to CNTF-expressing cells in the spinal cord and as a result of cell death in the olfactory bulb. Spinal motor neurons extend their axons out of the cord to innervate peripheral targets, such as hindlimb muscle, where they are exposed to physical stress from muscle activity. Masu *et al.* (13) have proposed that this type of low-level trauma resulting from normal motor function results in the leakage of CNTF from Schwann cells that surround these motor fibers. CNTF can be transported retrogradely from the sciatic nerve in the periphery into the spinal cord by motor neurons after injury (32). As we suggested above, the effect of CNTF overexpression in the olfactory bulb may be due to the release of the ligand from glial cells as a result of the ongoing cell death of olfactory receptor neurons. Stöckli *et al.* (29) have demonstrated that within the bulb, CNTF is expressed by glial cells that are in close association with the axons of the primary olfactory neurons that undergo turnover. It is plausible that the death of these neurons disrupts the associated glial cells resulting in the release of CNTF into the extracellular milieu.

How CNTF induces reactive gliosis is at present unclear. Whether CNTF acts directly or via other cell types to generate

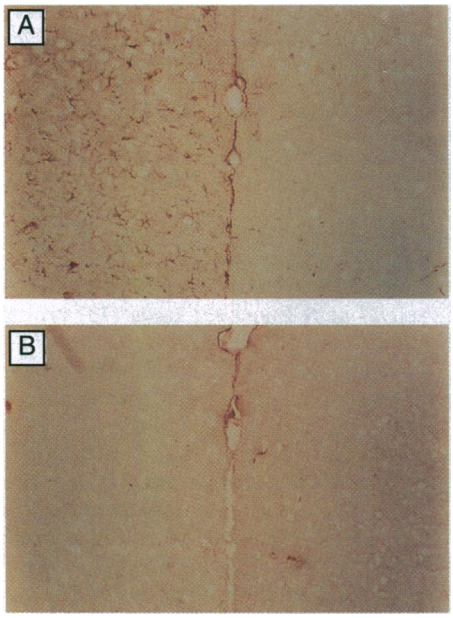

FIG. 3. CNTF induces widespread reactive gliosis when injected intracerebrally into wild-type mice. Mice receiving a single stereotactic injection of 0.36 μg of rhCNTF (2.5 mm anterior, 1.5 mm lateral, and 1.5 mm ventral relative to Bregma) develop many strongly staining $GFAP^+$ astrocytes in the ipsilateral hemisphere relative to the contralateral side (*A*). The same dose of CNTF, heated to inactivate the protein, did not produce an effect (*B*). The photomicrographs are representative of seven mice injected with active CNTF and three injected with inactivated protein. (×40.)

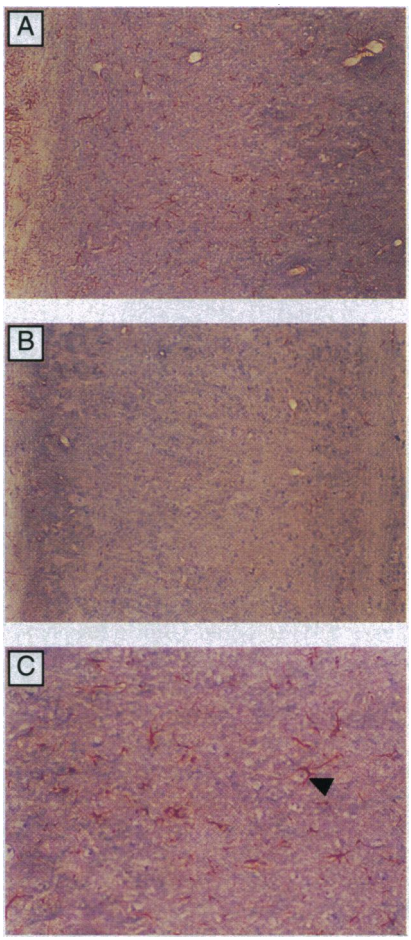

FIG. 4. Reactive astrocytes in the transgenic spinal cord. Staining with an anti-GFAP antibody reveals hypertrophied astrocytes throughout the gray matter of the transgenic spinal cord (*A*), relative to an equivalent section of the wild-type spine (*B*). In the transgenic mouse, intensely staining astrocytes appear to be wrapped around neuronal cell bodies (*C*), indicated with an arrowhead. These cells are also visible in the lower-power photomicrograph of the transgenic mouse (*A*) but not of the wild-type mouse (*B*). The spinal sections shown in this figure were cut longitudinally and are 20 μm thick. (*A* and *B*, ×40; *C*, ×80.)

reactive astrocytes remains a question. It is also unknown whether these CNTF-induced reactive astrocytes are derived from preexisting astrocytes or glial precursors. CNTF promotes the expression of GFAP and the formation of an astrocytic morphology in cultured glial progenitor cells (5). One possibility is that an analogous *in vivo* glial progenitor cell type may similarly respond to CNTF by differentiating into a cell expressing high levels of GFAP. Alternatively, CNTF may bind directly to mature committed astrocytes signaling an alteration in cellular morphology that is characteristic of the reactive astrocytes. Supporting data for such a model has recently been published (33). Hippocampal astrocytes possess the functional tripartite CNTF receptor complex so that exposure to CNTF leads to intracellular phosphorylation of the gp130 subunit. Also, physical injury to the cortex was shown to increase the synthesis of CNTF receptor α subunit in what appeared to be astrocytes, providing further suggestive evidence that CNTF may have a physiological function in signaling during an astrocyte-mediated injury response. Although it has not been shown whether microglia/brain macrophages jhave CNTF receptors, it is interesting to note that peritoneal macrophages have been shown to be devoid of them (33).

Reactive gliosis is manifested in a number of neurodegenerative diseases including Alzheimer, Huntington, multiple sclerosis, and AIDS-related dementia (14, 15). The function of reactive glia in the pathology of or recovery from neurological insult is not understood. As CNTF has been shown to support the survival and regeneration of injured neurons *in vivo*, it will be of interest to determine whether CNTF-activated glia and the process of gliosis affect this outcome. Because the *cntf* transgenic mice are predisposed to gliosis, they provide a useful model for probing the role of gliosis in responding to lesions in the CNS.

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