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CILIARY NEUROTROPHIC FACTOR IN THE OLFACTORY BULB OF RATS AND MICE

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Abstract—Ciliary neurotrophic factor (CNTF) is primarily regarded as an astrocytic lesion factor, promoting neuronal survival and influencing plasticity processes in deafferented areas of the CNS. Postnatal loss of neurons in CNTF-deficient mice indicates a function of the factor also under physiological conditions. In the olfactory bulb, where neurogenesis, axo- and synaptogenesis continue throughout life, CNTF content is constitutively high. The cellular localization of CNTF in the rat olfactory bulb is not fully resolved, and species differences between mouse and rat are not yet characterized. In the present study, four different CNTF antibodies and double immunolabeling with specific markers for glial and neuronal cells were used to study the cellular localization of CNTF in rat and mouse olfactory bulb. Specificity of the detection was checked with tissue from CNTF-deficient mice, and investigations were complemented by immunolocalization of reporter protein in mice synthesizing β-galactosidase under control of the CNTF promoter (CNTF lacZ-knock-in mice). In both species, CNTF localized to ensheathing cell nuclei, cell bodies and axon-enveloping processes. Additionally, individual axons of olfactory neurons were CNTF immunoreactive. Both CNTF protein content and immunoreaction intensity were lower in mice than in rats. Scattered lightly CNTF-reactive cells were found in the granular and external plexiform layers in rats. Some CNTF-positive cells were associated with immunoreactivity for the polysialylated form of the neural cell adhesion molecule, which is expressed by maturing interneurons derived from the rostral migratory stream. In CNTF lacZ-knock-in mice, β-galactosidase reactivity was found in ensheathing cells of the olfactory nerve layer, and in cells of the glomerular, external plexiform and granular layers. The study proves that CNTF is localized in glial and neuronal structures in the rodent olfactory bulb. Results in mice provide a basis for investigations concerning the effects of a lack of the factor in CNTF-deficient mice.

Key words: ensheathing cells, olfactory neurons, neurogenesis, immunocytochemistry, glial fibrillary acidic protein, neural cell adhesion molecule.

Neurotrophic factors are essential not only for normal embryonic development of the complex and intricate network of neural cells composing the nervous system, but are also critically involved in maintenance and repair of the adult nervous system (Weisenhorn et al., 1999). Ciliary neurotrophic factor (CNTF) has gained increasing attention for its possible actions in physiological maintenance and injury response in the adult CNS (e.g. Ip and Yancopoulos, 1996). CNTF is a member of the four α-helical cytokine family including interleukin 6, leukemia inhibitory factor, and others (Sendtner et al., 1994; Weisenhorn et al., 1999). Previous studies have indicated that its synthesis is restricted to glial cells in the postnatal nervous system (Stöckli et al., 1991; Dobrea et al., 1992) with highest concentrations in Schwann cells, astrocytes of the optic nerve, and in the olfactory bulb (Stöckli et al., 1991; Guthrie et al., 1997). Astrocytic CNTF expression is also found in fiber tracts such as the fornix, but is low in central nervous gray areas (Dallner et al., 2002). Here, it is drastically up-regulated after mechanical or ischemic lesions, both at the lesion site and in the deafferented target area of the lesioned neurons (Guthrie et al., 1997; Lee et al., 1997). Overexpression of CNTF in transgenic mice results in gliosis reminiscent of gliotic reactions after lesions or in neurodegenerative diseases (Winter et al., 1995). Mice lacking CNTF initially thrive, but show enhanced motoneuron degeneration after a few months (Masu et al., 1993). These observations have led to the suggestion that endogenous CNTF may be involved in the protection and/or repair of injured neurons and of axonal projections subjected to mechanical stress (peripheral motor axons, optic nerve), continuous regeneration (olfactory system) or intense plastic remodelling (septo-hippocampal system; Stöckli et al., 1991; Winter et al., 1995; Dallner et al., 2002). Consequently, therapeutic application of CNTF has been used in animal models of neurodegenerative diseases, and has, for instance, been shown to effectively protect striatal neurons in a model of Huntington's disease (Emerich et al., 1998; Mittoux et al., 2000). Two percent to

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Abbreviations: a-CNTFm, monoclonal mouse–anti-rat ciliary neurotrophic factor; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; CNTFRα, ciliary neurotrophic factor receptor α; DAB, 3-3'-diaminobenzidine; FGF2, basic fibroblast growth factor; GA, glutaraldehyde; ga-CNTFp, polyclonal goat–anti-ciliary neurotrophic factor; GFAP, glial fibrillary acidic protein; GOD, glucose oxidase; ir, immunoreactive; NCAM, neuronal cell adhesion molecule; NDS, normal donkey serum; NGS, normal goat serum; NiDAB, nickel-intensified 3-3'-diaminobenzidine; NLS, nuclear localization signal; OMP, olfactory marker protein; ON, olfactory neuron; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PSA, polysialylated; ra-CNTFp, polyclonal rabbit–anti-rat ciliary neurotrophic factor.

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3% of the human population are CNTF deficient (Takahashi et al., 1994; Thome et al., 1997). Recent reports indicate an involvement of the CNTF deficiency in neurological disease (Giess et al., 1998, 2002). Thus, elucidation of the role of CNTF in plasticity of the nervous system may be of considerable importance not only for therapeutic strategies, but also for resolving the etiology of neuropsychiatric diseases.

The olfactory system is unique among telencephalic nervous tissues in that plasticity is continuous throughout adult life. Primary sensory neurons of the olfactory (ONs) and vomeronasal epithelium are continually lost owing to normal turnover or to cell death induced by toxic or infectious agents in the air, and are replaced by asymmetric division of precursor cells in the epithelium (e.g. Martinez-Marcos et al., 2000; Mackay-Sim and Chuah, 2000). Axons of the newly formed neurons extend into the olfactory or accessory olfactory bulb, where they ramify in specific glomeruli and form synaptic contact with projection and interneurons. Moreover, interneurons of the olfactory bulb are newly generated throughout life: neurogenesis takes place in the forebrain subventricular zone, and developing neurons migrate along the "rostral migratory stream" into the olfactory bulb (Luskin, 1993; Bonfanti et al., 1997), where they populate different layers, differentiate into interneurons, and are apparently integrated into existing neuronal circuits (Gheusi et al., 2000; Carlen et al., 2002).

The olfactory system thus provides a model in which mechanisms involved in neuron generation, survival, differentiation, migration, axon growth and targeting, and synaptogenesis in the adult brain can be studied under physiological conditions. In particular, the role of specific growth factors such as CNTF can be investigated, for instance by investigating gene knockout mice. A necessary first step for these investigations is to determine the exact cellular localization of the neurotrophic factor in the different parts of the olfactory system. In the rat olfactory bulb, synthesis of CNTF in the olfactory nerve layer, presumably in glial cells, has been documented (Stöckli et al., 1991; Guthrie et al., 1997). Other investigations indicated CNTF localization in a number of neuronal cell types of olfactory epithelium and/or bulb (Henderson et al., 1994; Buckland and Cunningham, 1999). Data on the localization of CNTF in the mouse olfactory system have not been reported yet. Growth factor expression and synthesis may differ among rodent species (Mackay-Sim and Chuah, 2000), and thus results in the rat cannot be extrapolated to the mouse. Knowledge of the situation in mouse, however, is a necessary prerequisite for investigations in knockout animals.

Therefore, in the present study, carefully controlled immunoreactions with different CNTF antibodies were carried out to resolve the somewhat controversial issue of cellular and subcellular localization of CNTF in the rat olfactory and accessory olfactory bulb. CNTF-immunoreactive (ir) cells were identified by double immunolabeling with markers for specific cell types. The results were compared with the immunolocalization of CNTF in the mouse, specificity was checked in homozygous CNTF−/− mice, and results in wildtype (CNTF+/+) mice were complemented by investigations of the localization of β-galactosidase in mice genetically engineered to express the enzyme under the CNTF-promoter.

EXPERIMENTAL PROCEDURES

Animals

Rats. Adult male and female Wistar rats were bred in our animal facility or purchased from Charles River (Sulzfeld, Germany).

Wild type (CNTF+/+) and CNTF-gene knockout (CNTF−/−) mice. CNTF−/− mice (Masu et al., 1993) were continuously bred in our animal facility and backcrossed to C57/Bl6 mice supplied from Charles River at least every third generation. Heterozygous mice were genotyped by Southern blot analysis as described (Masu et al., 1993) and intercrossed to produce F1 offspring which contained both mice with CNTF−/− and CNTF+/+ genotype. These mice were either directly used or further propagated for one generation for production of animals used in this study. The mice were routinely screened for viral infections and were essentially free of infections with mouse hepatitis virus, reovirus type 3, Theiler's encephalomyelitis virus, pneumonia virus of mice, Sendai virus, and minute virus of mice.

CNTF-lacZ knock-in mice. The bacterial β-galactosidase protein coding sequence was introduced into the CNTF locus to facilitate the identification of cells expressing low levels of CNTF. The gene targeting construct consisted of 2.4 kb 5' flanking fragment and a 4.5 kb 3' flanking fragment. The nuclear localization signal (NLS) from the small T antigen (Kalderon et al., 1984) was cloned in front of the lac Z protein coding region in order to localize the reporter protein to the nucleus. The pGK-neo-polyA selection marker was cloned downstream of NLS-lacZ. The NLS-lacZ/neo cassette was introduced into the first exon of the CNTF gene between restriction sites *Spe*I and *Nhe*I such that the CNTF translation start site was deleted. Homologous recombination led to the disruption of the CNTF gene. Therefore, homozygous CNTF-lacZ knock-in mice are CNTF-gene knockouts. Breeding, backcrossing, and genotyping of the mice was carried out as described above. All experiments were done conforming to the guidelines on the ethical use of animals according to the German Law for the Protection of Animals, and were designed to minimize the number of animals used and their suffering.

Western blots

Rats and CNTF-wild type mice were decapitated under anesthesia, the sciatic nerves and the olfactory bulbs were dissected and tissue extracts were prepared after homogenization using a glass-glass homogenizer as described previously (Saadat et al., 1989). Protein concentration was determined using the Coomassie blue-based protein assay (Bradford, 1976; BioRad, Munich, Germany). Ten micrograms of protein of the sciatic nerve extracts and 50 μg of the olfactory bulb extracts were run per lane on 12% polyacrylamide gels under reducing conditions. Recombinant rat CNTF (Squinto et al., 1990) was coelectrophoresed at appropriate concentrations in separate lanes. Blotting and immunodetection was carried out as described previously (Stöckli et al., 1991), except that a rabbit polyclonal antiserum against CNTF (dilution: 1:2000; Masu et al., 1993; see below) was used for detection.

Immunocytochemistry

Tissue preparation. Rats (n=20) were anesthetized and, after a short pre-rinse, perfused transcardially with either 4% paraformaldehyde (PFA) in 0.01 M phosphate-buffered saline for

15 min (PBS; Fix I;); 4% PFA in 0.2% sodium acetate pH 6.5 for 10 min followed by 20 min 4% PFA in 0.1 M sodium carbonate–bicarbonate at pH 11 containing 0.2% glutaraldehyde (GA; Fix II; Berod et al., 1981; Liposits et al., 1986); or 4% and 15% saturated picric acid in 0.1 M phosphate buffer pH 7.2 containing 0.08% GA for 10 min (Fix III). Adult mice of both sexes (wild type: $n=5$; CNTF−/−: $n=4$; homozygous and heterozygous CNTF-lacZ knock-in mice: $n=2$ each) were perfused using Fix I for 10 min. After perfusion, the olfactory and accessory olfactory bulbs were dissected and postfixed for 3 h at room temperature or overnight at 4 °C. Tissue for light microscopy was then washed in PBS, infiltrated successively with 10% and 20% sucrose in PBS, frozen in liquid nitrogen-cooled isopentane, and stored at −80 °C. Sections were cut either using a cryostat (10–30 μm) or, after gradual thawing to room temperature, using a vibratome (30–50 μm; Leica VS, Leica Instruments, Bensheim, Germany). Tissue for electron microscopy was washed in PBS after postfixation and immediately vibratome sectioned. Vibratome sections were either directly processed for immunocytochemistry or stored in cryoprotective solution at −40° until needed.

Antibodies. Four different CNTF antibodies were used for the experiments: polyclonal rabbit–anti-rat CNTF (ra-CNTFp; IgG fraction; Masu et al., 1993; dilution 1:500–1:5000), monoclonal mouse–anti-rat CNTF (a-CNTFm; 1:250–1:1000), polyclonal goat–anti-CNTF (ga-CNTFp; R&D, Wiesbaden, Germany; dilution 1:500) and polyclonal chicken-anti-CNTF (Promega, Mannheim, Germany). Goat-anti-olfactory marker protein (OMP)-antiserum was a gift of Dr. F. Margolis, Baltimore, USA. Other antisera and antibodies used were: rabbit–anti-glial fibrillary acidic protein (GFAP; Dako, Hamburg, Germany; dilution 1:200), monoclonal mouse-anti-GFAP (Sigma, Deisenhofen, Germany; dilution 1:20,000), monoclonal mouse–anti-neural cell adhesion molecule (NCAM; Sigma; 1:250), monoclonal mouse–anti-polysialated (PSA) NCAM (gift of Dr. G. Rougon, Marseille or AbCys, Paris, France; 1:500), polyclonal rabbit–anti-β-galactosidase (Sigma, 1:200). Lower dilutions were used for immunocytochemistry on mounted cryostat and semithin sections, higher dilutions for reactions using free-floating cryostat and vibratome sections. Secondary antisera for immunoenzyme histochemistry were biotinylated goat–anti-mouse, goat–anti-rabbit or rabbit–anti-goat IgG (Vector, Burlingame, CA, USA, or Rockland, Gilbertsville, PA, USA or Dako; dilution 1:300–500). Secondary antisera for immunofluorescence were Cy2 or Cy3-labeled goat–anti-rabbit, goat–anti-mouse IgG, goat–anti-chicken IgY, donkey–anti-rabbit and donkey–anti-goat IgG (Dianova, Hamburg, Germany; Amersham, Freiburg, Germany; Abcam, Cambridge, UK; dilution 1:600).

Immunoreactions for light and fluorescence microscopy. Ten to 14 μm cryostat sections were thawed onto Superfrost slides (Menzel, Braunschweig, Germany) and dried under a cold stream of air for at least 3 h. Sections were washed in PBS, preincubated in PBS with 2% normal goat serum (NGS), normal donkey serum (NDS; both Sigma) or 0.25% λ-carrageenan (for incubations with primary antibodies generated in goat; Sigma) and 1% Triton X-100 for 1–2 h, and subsequently incubated in the appropriate dilution of the primary antibody (single labeling) or combination of antibodies (double labeling) in 1% NGS, 1% NDS or 0.25% λ-carrageenan, 0.5% Triton X-100 in PBS (incubation buffer) containing 0.05% NaN_3 overnight at 4 °C in a humid chamber. After washing in PBS, sections for single labeling immunoenzyme histochemistry were incubated in the appropriate biotinylated secondary antibody in incubation buffer overnight at 4 °C, washed in PBS, and incubated with streptavidin–biotinylated peroxidase complex (Dako) in PBS for 2–3 h. After brief washing in PBS, antigen localization was visualized using the 3-3'-diaminobenzidine–glucose–oxidase method with or without nickel intensification as described (DAB-GOD or NiDAB-GOD-method; Zaborszky and Heimer, 1989; Asan 1998). Sections for single or double labeling immunofluorescence were incubated in the appropriate secondary antibodies in incubation buffer for 3 h at RT or overnight at 4 °C, washed, mounted in medium containing an antifading agent, and observed in an Olympus BHS microscope or in a Zeiss LSM 5 confocal microscope.

Thicker cryostat sections (40 μm) and vibratome sections were incubated free-floatingly according to the protocol detailed for the cryostat sections, with some modifications: 3 h preincubation, incubation in primary antibodies for 48–72 h at 4 °C, incubation in secondary antibodies overnight at 4 °C. Antigen detection was as described above. Finally, sections were mounted on Superfrost slides. For immunofluorescence observation, sections were immediately coverslipped with medium containing antifading agent. For immunoenzyme histochemistry, sections were dried, dehydrated, cleared in xylene and coverslipped in Depex.

Immunoreactions for electron microscopy. Vibratome sections from tissue fixed with Fix II and Fix III were treated as described above with minor modifications: preincubation and incubation buffers contained none or 0.04% Triton X-100, respectively. After detection of the antigen with the NiDAB-GOD method, sections were washed in PBS, osmicated in 1% OsO_4 in PBS for 1 h, washed again in PBS, dehydrated in graded ethanol and finally flat embedded in Epon between sheets of Aclar foil (Plano, Wetzlar, Germany). After polymerization, sections were observed under the light microscope, photographed, and areas of interest were cut out and reembedded onto empty Epon blocks. Ultrathin sections were prepared, floated on formvar-coated grids, contrasted with uranyl acetate and lead citrate (Reynolds, 1963) and observed in a LEO 912 AB (Leo Elektronenmikroskopie, Oberkochen, Germany).

Controls. For the rabbit–anti-CNTF antiserum, preadsorption controls were carried out. The antiserum was incubated overnight at 4 °C with gentle agitation with an excess of recombinant rat CNTF. After centrifugation, the supernatant was used for immunoenzyme histochemistry as described above on rat tissue in parallel to immunoreactions with the non-preadsorbed antiserum. Specificity was further checked for all antibodies used in every experiment by omitting the primary antibodies from the reaction sequence on some sections.

RESULTS

Western blots

Bands with the same electrophoretic mobility as recombinant rat CNTF (approximately 23 kD) were detected in the sciatic nerves and olfactory bulbs of both rats and mice. Fig. 1 shows representative results. Semiquantitative analysis indicated that while the sciatic nerves of mice appeared to contain relatively more CNTF protein than those of rats, the relative CNTF content in olfactory bulbs was higher in rats than in mice.

Immunohistochemistry: CNTF immunodetection using different antibodies, influence of tissue pretreatment and detection methods

In rat tissue fixed with Fix I, the polyclonal rabbit and goat antisera and the monoclonal mouse antibody gave good labeling signals regardless of the detection protocol used. The highest immunoreaction intensity was seen with the polyclonal rabbit antiserum. The chicken antiserum gave overall weak immunoreactivity. Background staining was very high for the chicken antiserum, and was higher for all polyclonal antisera than for the

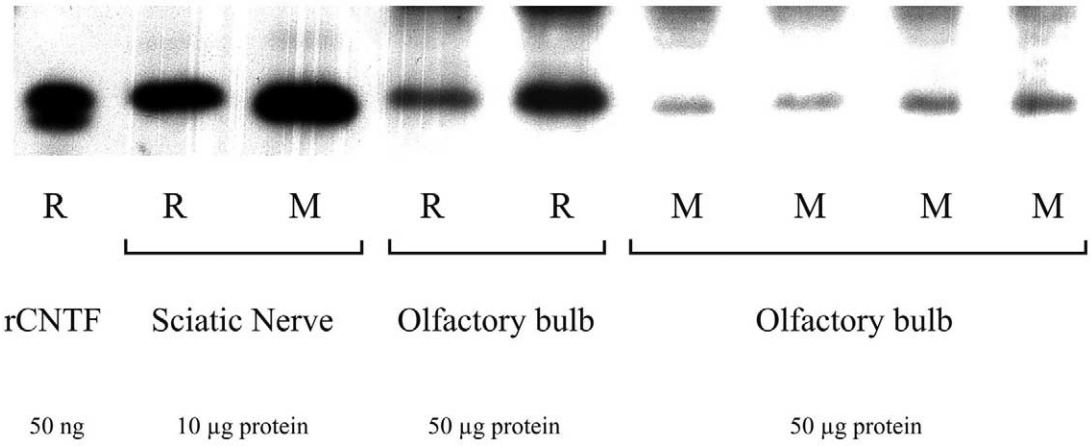

Fig. 1. Western blot of CNTF in rat (R) and mouse (M) sciatic nerve and olfactory bulb tissue protein extracts and of recombinant rat CNTF (rCNTF).

monoclonal antibody. The labeling patterns for the different antibodies in the olfactory bulb were identical for most cell types (cf. Figs. 2, 3) with one exception: in mitral cells of the olfactory bulb light immunolabeling was seen using the rabbit antiserum (Fig. 2a) and also using the chicken antiserum, but not using the goat and the monoclonal mouse antiserum. Since mitral cell staining using the rabbit antiserum could not be abolished by preadsorption with an excess of recombinant rat CNTF, it was considered unspecific. All other cell types described below as specifically CNTF-ir displayed labeling using all polyclonal antisera and the monoclonal CNTF antibody. The chicken antiserum gave very low signal-to-noise ratio. Using stronger fixatives (Fix II and III) or, in particular, immunolabeling for electron microscopy, the principal pattern of labeling was as described, but the labeling intensity was reduced.

In mouse tissue, the monoclonal CNTF antibody did not show specific labeling. The labeling patterns for the rabbit, goat and chicken polyclonal antisera were identical; the immunostaining intensity was highest using the rabbit antiserum and again very low using the chicken antiserum. Specificity of the CNTF detection was checked by comparing the immunoreactions using the rabbit antiserum in sections from wild type (CNTF+/+) mice with preadsorption controls and with staining patterns in sections from CNTF−/− mice. Only those structures were considered specifically labeled which showed labeling for all three polyclonal antisera and did not display labeling in the preadsorption controls or in the knockout mice.

CNTF immunoreactivity in olfactory and accessory olfactory bulb cell types

Rats. In the nerve layer of the olfactory bulb, intense immunolabeling for CNTF was found in many cells with ramifying processes (Figs. 2–6). Additionally, CNTF immunoreactivity was localized in fine varicose fibers in the olfactory nerve layer (Figs. 2c–e, 4a, 5a) which often could be observed to run into individual olfactory glomeruli, where they ramified extensively (Figs. 2–5). Occasionally, glomeruli were entirely filled by CNTF-ir fibers (Fig. 2c), and these fibers were detected in consecutive sections immunoreacted with different antibodies (Fig. 3a, b).

Double immunofluorescence labeling with GFAP showed colocalization with CNTF in nerve layer cells, confirming that these are the olfactory nerve ensheathing cells (Fig. 4a). All identified ensheathing cells were CNTF-ir. Staining was characteristically found both in the nuclear area and in the cytoplasm, extending into the fine, GFAP-ir processes dividing the fila olfactoria into axon bundles (Figs. 4a, 5a, b). CNTF/GFAP-ir processes of ensheathing cells did not extend into the core of the glomeruli (Fig. 4b). Semithin sections and electron microscopy confirmed the observation that CNTF was localized within the nuclei and small processes of ensheathing cells (Fig. 6).

GFAP-ir astrocytes of the glomerular layer, which extend their processes into glomeruli, did not display CNTF immunoreactivity (Fig. 4b, c). CNTF-ir intraglomerular fibers also lacked GFAP immunoreactivity, proving that they did not represent glial processes (Fig. 4c). The fibers, on the other hand, displayed NCAM immunolabeling, which preferentially detects olfactory neuronal axons (Miragall et

Abbreviations used in the figures

AGL	accessory glomerular layer
AOB	accessory olfactory bulb
CNTF-lacZ	CNTF-lacZ knock-in
EPL	external plexiform layer
FO	Fila olfactoria
GL	glomerular layer
GrL	granular layer
LOT	lateral olfactory tract
ML	mitral cell layer
ONL	olfactory nerve layer
PML	plexiform and mitral cell layer
VNL	vomeronasal nerve layer

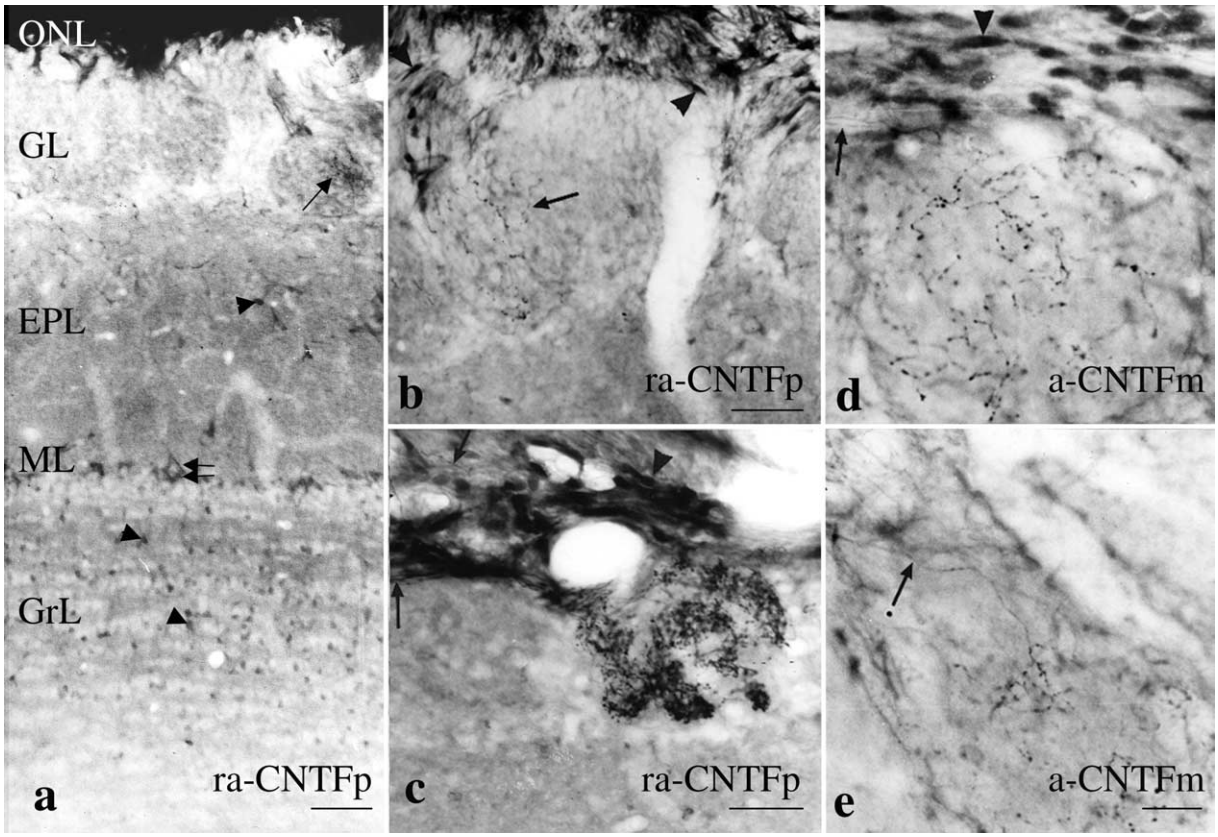

Fig. 2. Immunodetection of CNTF in rat olfactory bulb vibratome section using ra-CNTFp (a–c) and a-CNTFm (d, e). The dense immunoreaction in the ONL in (a) is due to labeling of numerous cells (arrowheads in b–d). Narrow, varicose CNTF-ir fibers (arrows in a–e) course from the ONL into individual glomeruli, where they ramify profusely. Occasionally, a single glomerulus is entirely filled with CNTF-ir fibers (c). Additionally, CNTF-ir cells are observed in the GrL and EPL (arrowheads in a). Staining of large cells in the ML (double arrows in a) is observed using ra-CNTFp. Bars in (a–c)=50 μm, in (d, e)=20 μm.

al., 1988; Fig. 5a–f). Additionally, OMP, which is expressed exclusively by mature ONs and their axons (e.g. Mackay-Sim and Chuah, 2000) was colocalized in CNTF-ir intraglomerular fibers (Fig. 5g–i).

In strongly immunoreacted sections, lightly CNTF-ir cells were found in the granular and, occasionally, in the external plexiform and glomerular layers of the bulb (Figs. 2a, 7). Labeling intensity was highest over the nuclei and very faint in the cytoplasm, only rarely extending into cell processes. Thus, in CNTF single labelings it was not possible to identify whether these ir cells represented astrocytes or neuronal cell types (e.g. periglomerular or granular cells, immature or maturing interneurons derived from the rostral migratory stream). Double labelings showed that the lightly CNTF-ir cells were not associated with GFAP-ir astrocytic cell processes (Fig. 7a). Double labeling with anti-PSA-NCAM, a marker for immature and maturing neurons (Bonfanti and Theodosis, 1994; Bonfanti et al., 1997; Peretto et al., 1999) revealed that some CNTF-ir cell bodies in the granular layer displayed faint but distinct PSA-NCAM immunoreactivity in the perikaryal plasma membrane (Fig. 7b). The shape of the cell bodies appeared to be oval, with main processes leaving the cell bodies at the two poles.

In the ependymal layer CNTF-ir cells were generally absent. A few lightly CNTF-ir cells with typical astrocytic morphology were observed in the region of the rostral migratory stream in forebrain and olfactory peduncle (not shown). In the olfactory peduncle, subpial CNTF-ir astrocytes extended CNTF-ir processes into the lateral olfactory tract (Fig. 8a). In the accessory olfactory bulb, ensheathing cells of the vomeronasal nerve layer were intensely labeled (Fig. 8b). Labeled fibers in glomeruli or labeled cells in other accessory bulb layers were not observed.

Mice. As in rats, CNTF immunoreactivity in CNTF+/+ mice was displayed by ensheathing cells of the olfactory nerve layers and by thin fibers which ran into individual olfactory bulb glomeruli where they ramified profusely (Fig. 9a, b). Immunoreaction intensity of ensheathing cells and the number of ir fibers was lower than in rat. No CNTF-ir cells could be identified in inner olfactory bulb layers (not shown). Ensheathing cells of the accessory olfactory bulb displayed CNTF immunoreactivity, but CNTF-ir fibers were not identified in its glomeruli (not shown).

CNTF-immunoreactivity was completely absent from olfactory bulbs of CNTF−/− mice (Fig. 9c). In both homozygous (CNTF deficient; Fig. 10a, b) and heterozygous

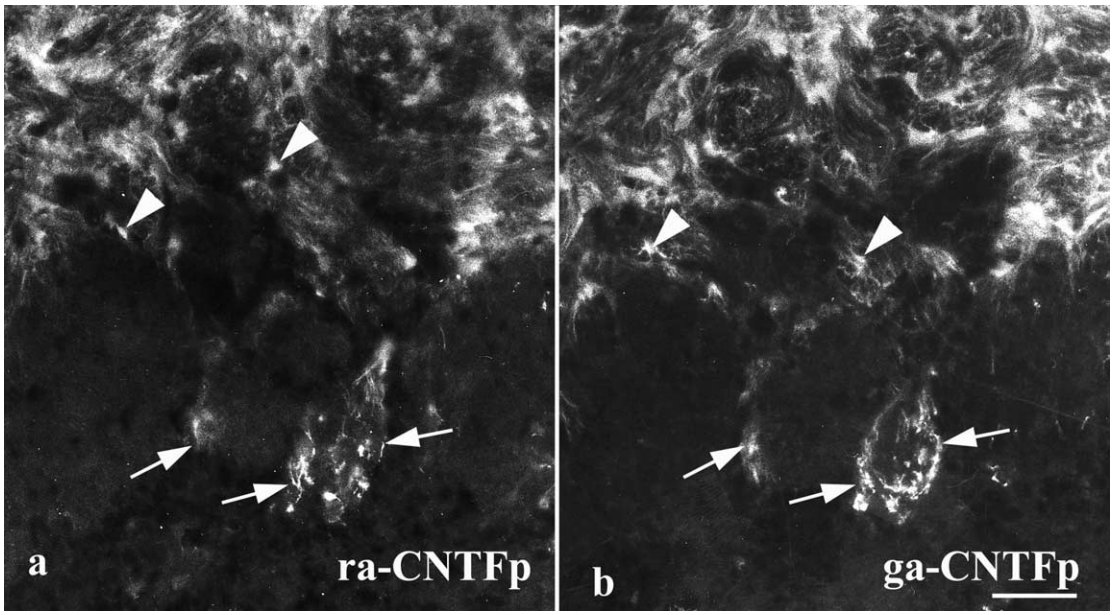

Fig. 3. Immunofluorescence labeling of CNTF in consecutive 14 μm cryostat sections of rat olfactory bulb using ra-CNTFp (a) and ga-CNTFp (b). In addition to CNTF-ir cells in the ONL (arrowheads), both antisera detect CNTF-ir fibers in an individual glomerulus (arrows). Bar=50 μm.

(CNTF±, lacZ−/+) CNTF-lacZ knock-in mice, immunoreactivity for β-galactosidase was found in the typical narrow nuclei of olfactory nerve layer ensheathing cells, indicating that synthesis of the reporter protein governed by the CNTF promoter takes place in these cells. Additionally, numerous nuclei of cells in the periglomerular, external plexiform and granular layers displayed β-galactosidase reactivity (Fig. 10c). The large nuclei of mitral cells were not stained. In CNTF+/+ mice, β-galactosidase staining was absent (Fig. 10d), indicating specificity of the reporter

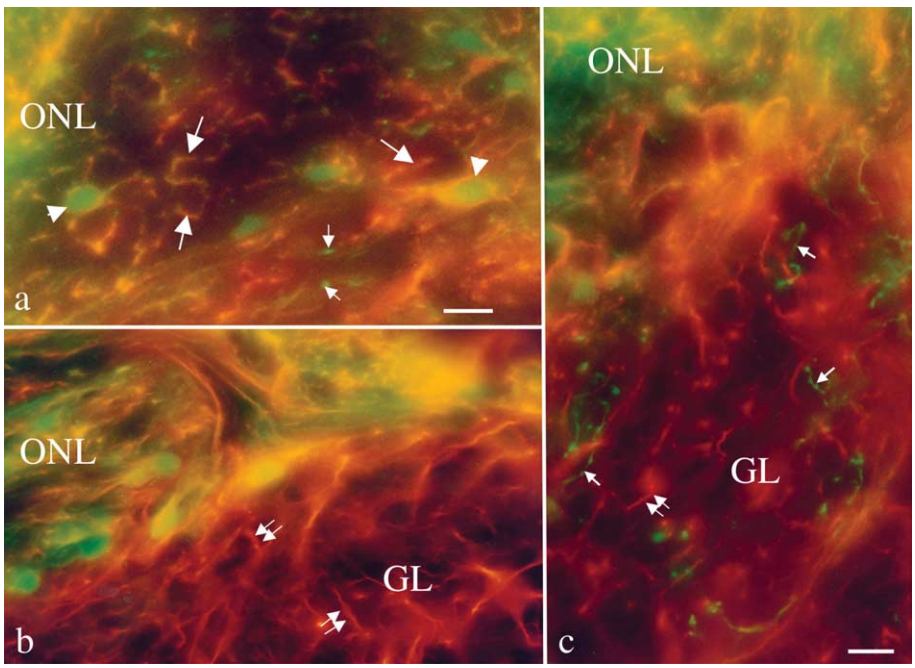

Fig. 4. Double immunofluorescence (a-CNTFm: green/a-GFAP: red) in 14 μm thick cryosections from rat olfactory bulb. (a) In transverse sections of fila olfactoria in the ONL, ensheathing cells, identified by GFAP colocalization (orange color) in their fine processes (large arrows), display intense CNTF immunoreactivity in their nuclear area (arrowheads). CNTF-ir narrow green fibers with varicosities are seen in longitudinally sectioned fila olfactoria (small arrows). (b, c) Double labeled processes of ensheathing cells in the ONL (orange) do not extend into glomeruli in the GL. Glial processes in the GL are red (double arrows), indicating that they arise from periglomerular non-CNTF-ir astrocytes. Varicose, branching fibers in individual glomeruli are exclusively CNTF-ir (green, small arrows in c). Bars=10 μm.

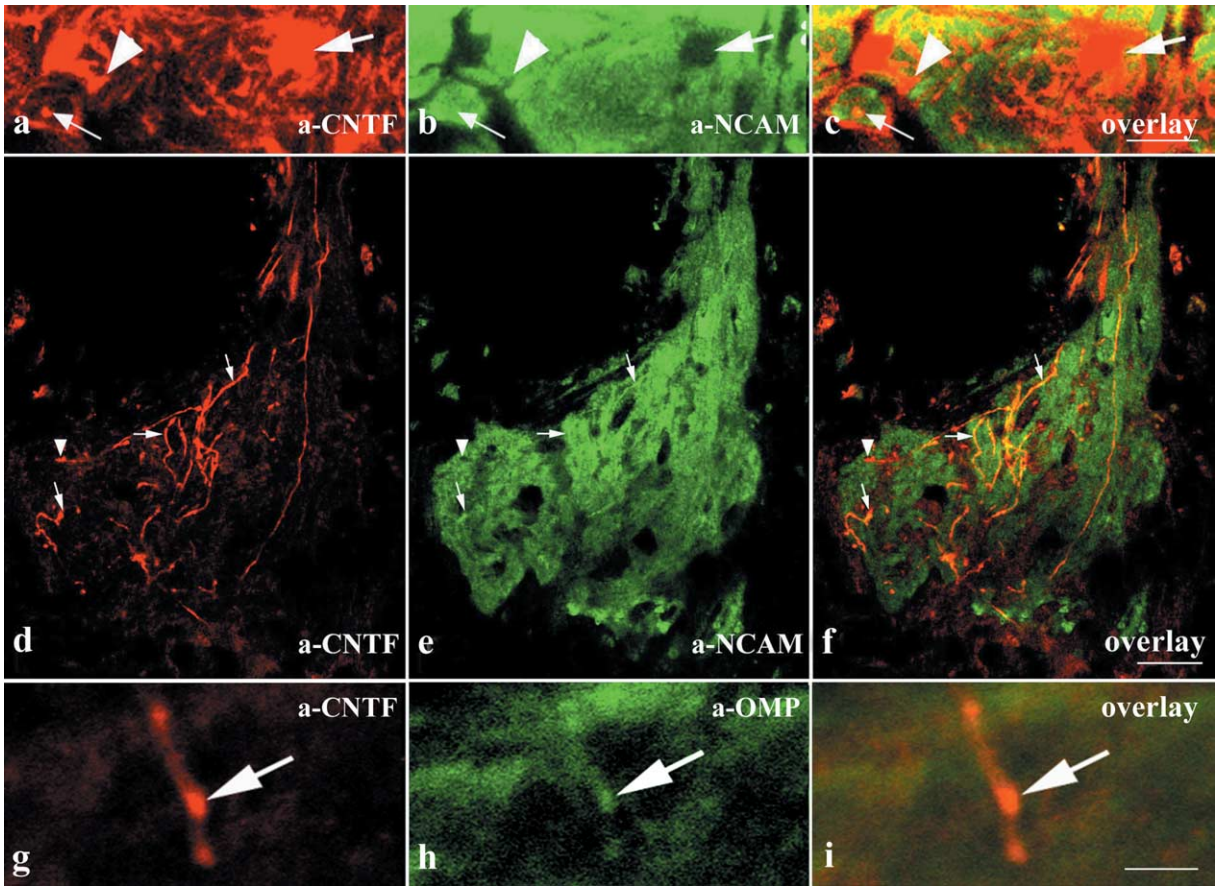

Fig. 5. Digital images of confocal scans of CNTF/NCAM (a–f) and of CNTF/OMP (g–i) vibratome sections of rat olfactory bulb. (a–c) In the olfactory nerve layer, bundles of olfactory axons are strongly NCAM-ir (b) while CNTF-ir ensheathing cell bodies (arrows) and processes (arrowheads) lack NCAM-immunoreactivity. Occasionally, a CNTF/NCAM double-labeled profile (small arrows) is observed in the axon bundles. (d–f) CNTF-ir fibers in individual glomeruli (arrows) are clearly NCAM-ir. A CNTF-ir, NCAM-negative profile at the rim of the glomerulus (arrowhead) most likely represents an ensheathing cell process and shows specificity of the detection. (g–i) CNTF-ir fibers (arrows) display light OMP-immunoreactivity. Bar in c=10 μm, in f=20 μm, in i=5 μm.

protein expression and detection in CNTF-lacZ knock-in mice.

For identification of the β-galactosidase-ir cells in the inner bulbar layers of CNTF-lacZ knock-in mice, immunoenzymatic and immunofluorescence labeling for β-galactosidase/GFAP and β-galactosidase/PSA-NCAM were carried out. To avoid effects of CNTF deficiency on transgene expression, these studies were performed on tissue from heterozygous animals. Immunoenzymatic detection of β-galactosidase gave a very high background which made identification of double labeled cells impossible. Confocal microscopy of GFAP/β-galactosidase immunofluorescence double labelings revealed that some of the β-galactosidase-ir nuclei were surrounded by bundles of GFAP-ir filaments, indicating that they were nuclei of astrocytes. Other β-galactosidase-ir nuclei, however, were clearly not associated with GFAP-ir cytoplasmic filament bundles (Fig. 10e–g). PSA-NCAM labeling in these mice was very weak. In some instances, PSA-NCAM/β-galactosidase double imunofluorescence labelings showed PSA-NCAM-reactive membranes in proximity to lightly β-galactosidase-ir nuclei, especially in the granular layer (not shown). However, a colocalization of the two antigens in the same cell could not be ascertained.

DISCUSSION

The results of the present study clearly demonstrate that both in the rat and mouse olfactory bulb, CNTF is localized in ensheathing cells of the olfactory and accessory olfactory nerve layers. Also, intense CNTF immunoreactivity is found in axons of individual olfactory receptor neurons ramifying in specific olfactory glomeruli. CNTF immunoreaction intensity in ensheathing cells and the relative content of CNTF protein in the olfactory bulb appears higher in rats than in mice. In olfactory bulb granular, plexiform, and periglomerular layers individual cells display light CNTF immunoreactivity in rats and CNTF promoter-driven reporter gene expression in CNTF-lacZ knock-in mice.

CNTF in the olfactory bulb: a controversial issue

Earlier studies have shown that in the normal adult rat brain, CNTF expression and protein content is highest in optic nerve and olfactory bulb. Immunocytochemistry indi-

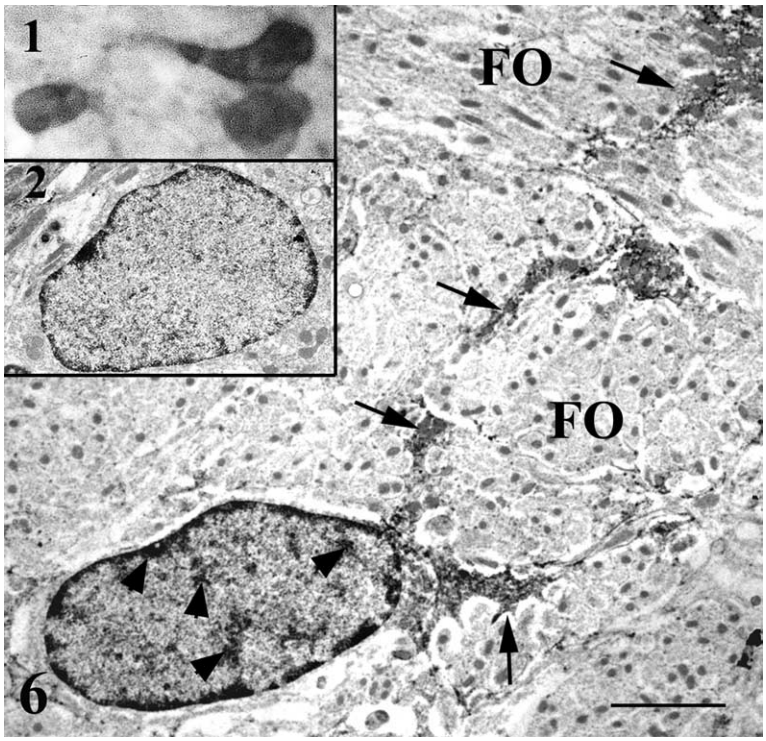

Fig. 6. An ensheathing cell of the olfactory nerve layer displays immunoreactivity in its processes (arrows) which ramify between bundles of FO. Imunoreaction product is additionally localized along the inner nuclear membrane and in clumps in the nuclear matrix (arrowheads). In the micrograph of a semithin section in inset 1, intense nuclear immunoreactivity of ensheathing cells is obvious; inset 2 shows the non-reactive nucleus of a periglomerular astrocyte for comparison with the labeled nucleus of the ensheathing cell. Bar=2 μm.

cated that in both areas subpopulations of glial cells synthesized the protein (Stöckli et al., 1991; Dobrea et al., 1992. Later, a localization of CNTF was postulated in all neuronal populations of the rat and mouse olfactory bulb

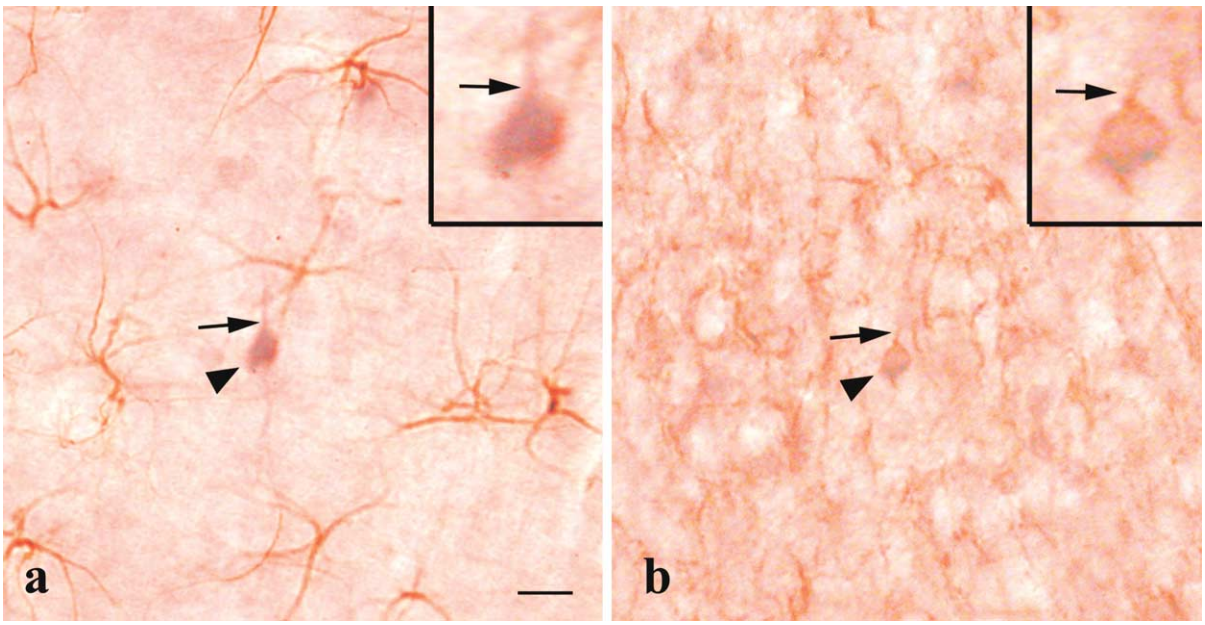

Fig. 7. Double immunolabeling (NiDAB/DAB-development, color intensities digitally enhanced) for CNTF/GFAP (a) or CNTF/PSA-NCAM (b) in vibratome sections of the granular layer of the rat olfactory bulb. (a) A dark gray CNTF-ir cell (arrowhead) is not associated with surrounding reddish-brown GFAP-ir astrocytes and astrocytic processes. The proximal process of the CNTF-ir cell (arrows, inset) lacks GFAP immunoreactivity. (b) Reddish-brown PSA-NCAM immunoreactivity labels membranes of numerous cellular processes in the granular layer. A CNTF-ir cell (arrowhead) displays reddish-brown PSA-NCAM immunoreactivity in the cell body membrane and proximal process (arrows, inset). Bar in a for a, b=20 μm.

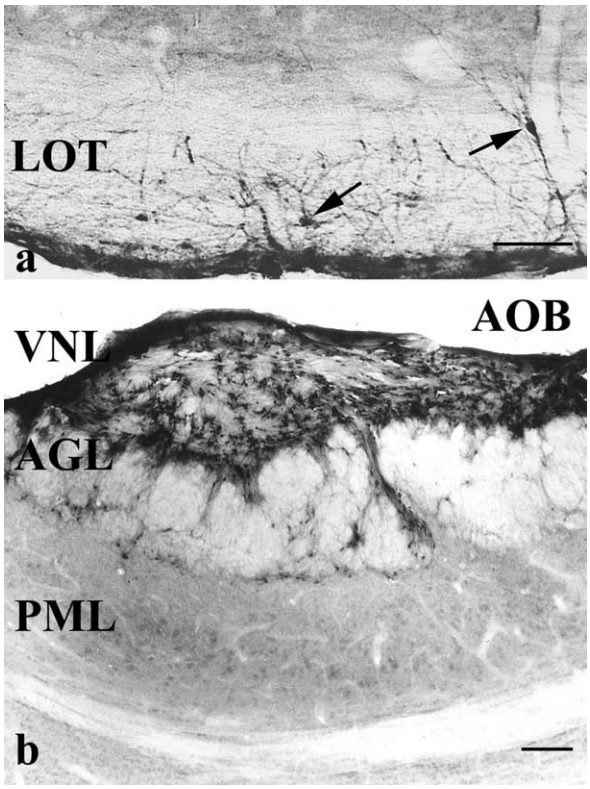

Fig. 8. Longitudinal vibratome sections of the olfactory peduncle (a) and AOB (b), reacted with a-CNTFm. (a) Subpial and blood-vessel-associated CNTF-ir astrocytes (arrows) extend long processes traversing the LOT. (b) Ensheathing cells of the VNL in the rat AOB are intensely CNTF-ir. Labeled fibers or other CNTF-ir cells are not observed. Bar in a=50 μm, in b=100 μm.

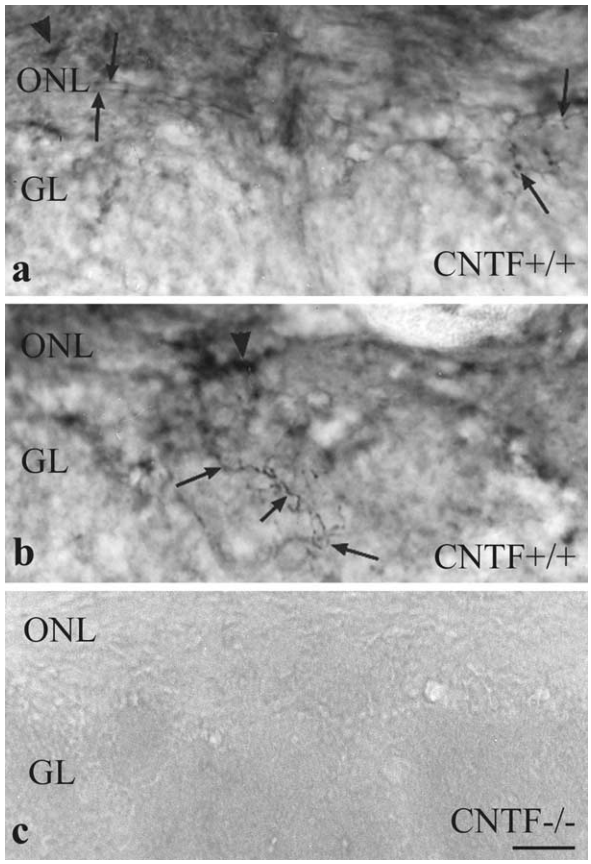

Fig. 9. Immunoreactions using ra-CNTFp on vibratome sections of mouse olfactory bulb. (a, b) CNTF-ir ensheathing cells (arrowheads) and fibers (arrows) in the wild type mouse. (c) Immunoreactivity is absent in sections from CNTF−/− mice. Bar=20 μm.

(Henderson et al., 1994). However, while CNTF production in cells of the olfactory nerve layer which does not contain neuronal cell bodies was confirmed by CNTF mRNA *in situ* hybridization, the hybridization signal density in the other, neuron containing layers of the olfactory bulb was not much higher than background (Guthrie et al., 1997). In a recent report, CNTF was immunolocalized to various neuronal cell types in the olfactory bulb including mitral cells, while staining in ensheathing cells of the nerve layer was not described (Buckland and Cunningham, 1999). *In vitro*, ensheathing cells from neonatal rat olfactory bulb were found to express both CNTF and its high affinity receptor (CNTF receptor α, CNTFRα; Wewetzer et al., 2001), while a stably transduced clonal ensheathing cell line, which expressed several *in vivo* markers of ensheathing cells, did not produce the factor (Goodman et al., 1993; Boruch et al., 2001). It was the aim of the present study to resolve the controversial issue of cellular localization of CNTF in the rat olfactory bulb, and to determine its localization in the mouse as a basis for further investigation into CNTF function in the olfactory system.

Methodological considerations

One essential prerequisite for the investigation was that specificity of the detection was checked in the most rigid manner possible. We used three different approaches to do this: first, we applied four different CNTF antibodies from different sources, compared labeling patterns, and defined as CNTF-ir only those elements that displayed labeling using all antibodies and were negative in preadsorption controls. Second, we checked for unspecific labeling in CNTF−/− mice. And third, we determined the localization of β-galactosidase in mice genetically engineered to express the reporter gene under the CNTF promoter. We expected to find, and indeed found, reporter gene expression in CNTF-lacZ knock-in mice in cells which were CNTF-immunolabeled in wild type mice, for instance in ensheathing cells, indicating that these cells are capable of producing CNTF.

CNTF in olfactory ensheathing cells

The largest contribution to the CNTF-content in the olfactory and accessory olfactory bulb is made by the ensheathing cells. These cells represent a type of central glia with properties both of astrocytes and of Schwann cells (Gudino-Cabrera and Nieto-Sampedro, 2000). Thus, ensheathing cells are GFAP-positive like astrocytes. They resemble Schwann cells since they are permissive for and even promote axonal growth (Ramon-Cueto and Valverde,

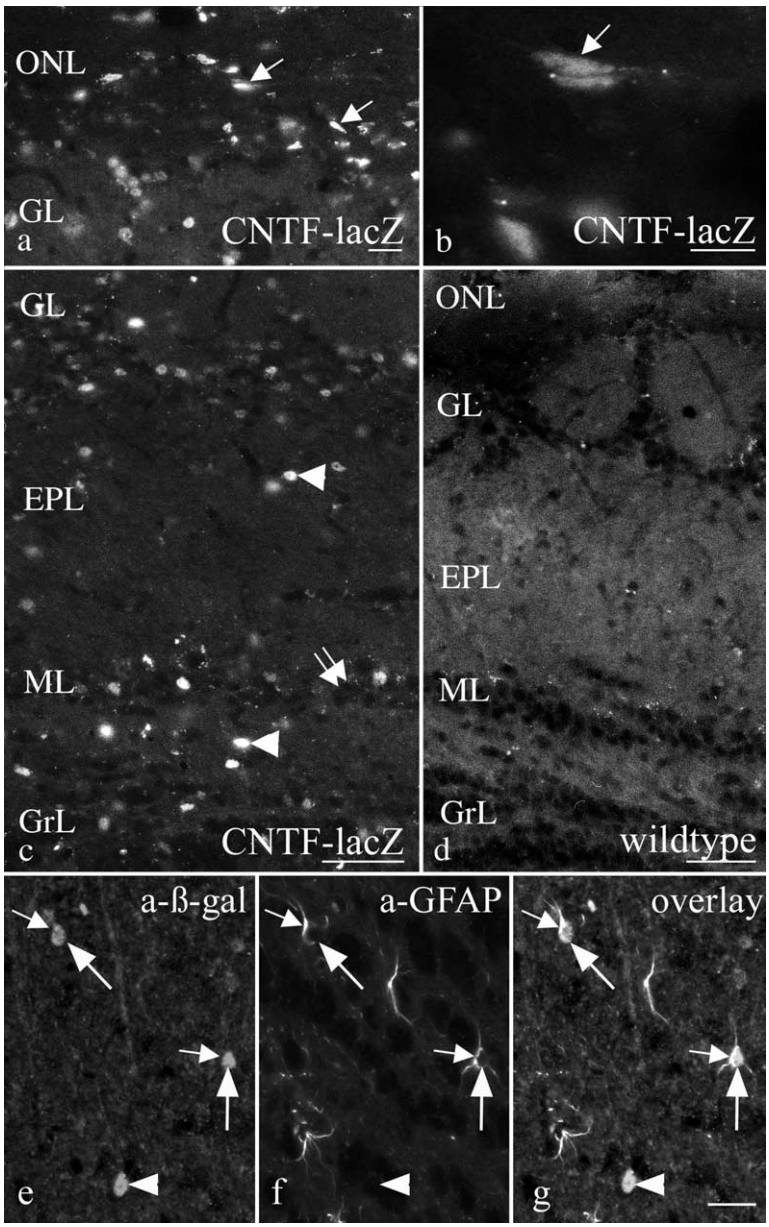

Fig. 10. Immunodetection of β-galactosidase in olfactory bulbs of CNTF-lacZ knock-in (a–c, e–g) and wild type (CNTF+/+; d) mice. (a) β-galactosidase staining is seen in nuclei of narrow cells in the ONL (arrows) which, at higher magnification (b; arrow) display the typical ensheathing cell morphology. Additionally, individual nuclei in the GL, EPL and GrL are labeled (a, arrowheads in c). The typical large nuclei of mitral cells in the ML (double arrows in c) are not labeled. β-galactosidase immunoreactivity is absent in wild type mice (d). (e–g) Confocal images of β-galactosidase/GFAP double immunolabeling in the granular layer. Two β-galactosidase-ir nuclei (large arrows) are associated with GFAP-ir filament bundles (small arrows), indicating that these cells are astrocytes. One β-galactosidase-ir nucleus (arrowhead) lacks surrounding GFAP immunoreactivity. Bar in a=20 μm, in b=10 μm, in c, d=50 μm, in g for e–g=0.20 μm.

1995; Ramon-Cueto and Avila, 1998; Kafitz and Greer, 1999; Ramon-Cueto et al., 2000). Ensheathing cell transplants have successfully been used for repair of spinal cord injuries. They bridge the injury-induced gap, promote axon growth across the lesion, and form myelin sheaths around regenerated or demyelinated axons (Imaizumi et al., 1998; Raisman, 2000; Barnett et al., 2000; Bartolomei and Greer, 2000; Franklin and Barnett, 2000; Lu et al., 2001, 2002). Axon growth in other regions of the adult rat CNS has also been shown to be induced by transplanted ensheathing cells (e.g. Smale et al., 1996; Perez-Bouza et al., 1998). Ensheathing cells supposedly support axon growth by synthesizing a specific permissive extracellular matrix (Tisay and Key, 1999), and growth factors (Mackay-Sim and Chuah, 2000; Bartolomei and Greer, 2000). Ensheathing cells have been shown *in vivo* to synthesize basic fibroblast growth factor (FGF2; Gall et al., 1994; Chuah and Teague, 1999), glial growth factor 2 (Chuah et al., 2000), and platelet derived growth factor-B (Sasahara et al., 1992; Kott et al., 1994). *In vitro*, synthesis of brain

derived neurotrophic factor (BDNF), of glial cell line-derived neurotrophic factor, and of CNTF has been documented (Mackay-Sim and Chuah, 2000; Wewetzer et al., 2001). Among these factors, CNTF is of special interest, since its application to chronic lesions of the spinal cord alone resulted in an increase in the number of injured supraspinal neurons which were able to regenerate and regrow their axons into a peripheral nerve graft (Ye and Houle, 1997). The effect of CNTF was higher than that of neurotrophin 3 and BDNF in this study. Thus, CNTF may be particularly important for the capacity of ensheathing cells to promote repair of spinal cord injuries. Physiologically, it may be required for olfactory and vomeronasal axon regrowth.

Subcellular localization of CNTF in ensheathing cells

In most immunodetection studies including the present one CNTF was observed in the cytoplasmic compartment (Stöckli et al., 1991; Lee et al., 1997). In addition, we found a nuclear localization of CNTF immunoreaction product in ensheathing cells. Recently, nuclear localization of CNTF in rat cortical astrocytes and other cell types was demonstrated, and it was suggested that a mechanism of facilitated transport was responsible for the nuclear translocation of the factor, indicating functional relevance of the translocation (Bajetto et al., 1999, 2000). Nuclear localization has also been demonstrated for FGF2, which, like CNTF, does not possess a hydrophobic signal peptide sequence for conventional secretion (e.g. Sendtner et al., 1994; Joy et al., 1997). For FGF2 it was suggested that nuclear accumulation of the factor and its receptor are operative events in mitotic activation and hypertrophy of human glial cells (Moffett et al., 1998). A gliotic effect was observed in olfactory bulbs of mice overexpressing CNTF (Winter et al., 1995). It will be interesting to investigate whether such “intracrine” effects could be a function of nuclear CNTF.

CNTF is also highly concentrated in the cytoplasm of the cell body and processes of ensheathing cells, and compelling evidence suggests that it is released from producing cells and acts as an autocrine or paracrine hormone via a membrane-bound tripartite receptor complex (Sleeman et al., 2000). The mechanisms of induction of CNTF expression and of its release are still elusive, although recent evidence points to a possible rapid secretion of proteins lacking a hydrophobic signal peptide by microvesicle shedding (MacKenzie et al., 2001). FGF2, like CNTF, is up-regulated in reactive astrocytes (Ip and Yancopoulos, 1996; Stachowiak et al., 1997; Levison et al., 2000). A loss of contact inhibition has been shown to enhance its expression (Moffett et al., 1996, 1998). Up-regulation of CNTF and CNTFRα is observed in astrocytes of deafferented hippocampal target areas after entorhinal cortex lesions (Guthrie et al., 1997; Lee et al., 1997), suggesting that induction of CNTF synthesis may be caused by rearrangement of axoglial contacts. Neuro–glial and neuro–neuronal intercellular adhesion contacts have been shown to play a decisive role in axon path finding, synaptogenesis and synaptic plasticity (Yoshihara and Mori, 1997; Shapiro and Colman, 1999; Benson et al., 2000). ONs express high amounts of NCAM (Miragall et al., 1988, present study), and both ONs and ensheathing cells synthesize cadherins, the integral membrane proteins of adherens junctions (Asan and Meyer-Stiegen, 1998; Lakatos et al., 2000). Rearrangement of axo-glial contacts in the olfactory nerve during degeneration and regrowth of olfactory axons could be one stimulus causing the constitutively high expression of CNTF in ensheathing cells.

CNTF in olfactory neuronal axons

A new and surprising finding of the present study was the presence of intense CNTF immunoreactivity in individual ON axons in the olfactory nerve and glomerular layers. Recently, a general CNTF immunoreactivity in cells of the ON lineage in the olfactory epithelium was reported (Buckland and Cunningham, 1999). Our findings confirm neuronal localization of the factor in the primary olfactory pathway. However, the fact that only a small proportion of olfactory axons are CNTF-ir argues against the localization of the factor in all ONs. In contrast, our data indicate that only a specific subset expresses and/or contains CNTF. This is in accordance with data by Stefanuto et al. (1995), who observed specifically strong transcription of the transgene in a mouse line expressing β-galactosidase under the CNTF promoter in some ONs and their axons. The characterization of CNTF-ir ONs is subject of further studies.

Other CNTF-ir cell types

CNTF-ir structures other than ensheathing cells and ON axons were only observed in the rat. These structures were only faintly immunolabeled. Their localization in the bulb suggests that these CNTF-ir cells may be subpopulations of interneurons and/or of glial cells. We could not find colocalization of GFAP in these CNTF-ir cells in rats, indicating that they were not mature astrocytes. Perikaryal plasma membranes of some CNTF-ir cells displayed faint but clear immunoreactivity for the polysialylated form of NCAM (PSA-NCAM), a marker for migrating and maturing interneurons derived from the subventricular zone neuroblasts which supplement the pool of interneurons in the granular and periglomerular layers of the main and accessory olfactory bulb (Bonfanti and Theodosis, 1994; Bonfanti et al., 1997; Peretto et al., 1999; Gheusi et al., 2000; Carlen et al., 2002). Since processes of CNTF/PSA-NCAM-ir cells were only lightly immunostained with either antibody, it was not possible to unequivocally identify the morphology of double labeled cells. However, the CNTF/PSA-NCAM-ir cells did not possess the bipolar morphology and intense PSA-NCAM labeling typical for migrating interneurons in the granular layer (Bonfanti and Theodosis, 1994). This observation, and the fact that only few CNTF-ir cells in inner bulbar layers were lightly PSA-NCAM-ir, may indicate that CNTF immunoreactivity characterizes a late maturation stage when the interneurons die or integrate into existing circuits.

In CNTF lacZ-knock-in mice, β-galactosidase immunoreactivity was found in numerous nuclei throughout the

layers, confirming CNTF promoter-initiated expression in additional bulbar cells. Some of these nuclei were associated with GFAP immunoreactivity, suggesting that even in heterozygous transgenic mice astrocytic CNTF promoter-driven expression was more active than in rats, where CNTF/GFAP double-labeled cells were absent, or than in wild type mice, where no CNTF-ir cells were found in inner bulbar layers (see above). Additionally, β-galactosidase-ir nuclei were present in transgenic animals that were clearly not associated with GFAP immunoreactivity. We performed β-galactosidase/PSA-NCAM double labelings to assess whether these cells may be maturing interneurons. However, PSA-NCAM staining in these mice was very weak, and confocal microscopy of double labelings did not show clear colocalization of the two markers. Thus, these β-galactosidase-positive, GFAP-negative cells in the inner bulbar layers remain to be identified.

In contrast to previous studies (Henderson et al., 1994; Buckland and Cunningham, 1999), we failed to find either specific CNTF immunoreactivity or CNTF promoter-driven transgene expression in mitral cells of rats and mice. In the olfactory peduncle, subpial astrocytes extending processes into the olfactory tract were intensely CNTF labeled, confirming the observation that astrocytes in tracts of long axonal projections (e.g. optic tract, fornix, capsula interna) display specifically high expression of this neurotrophic factor (Dallner et al., 2002).

Interspecies differences

In principle, cellular localization of CNTF was identical in rat and mouse olfactory bulbs. In mice, the CNTF content of the olfactory bulb as determined by Western blot, the immunoreaction intensity of ensheathing cells and the number of CNTF-ir ON axons were lower than in rats. The fact that transgene expression was found in cells in the inner layers of the bulb of CNTF lacZ-knock-in mice indicates that lack of detectable CNTF-ir cells in these layers in mice may be a problem of detection sensitivity.

CONCLUSION

CNTF is synthesized by glial and neuronal cells of the olfactory bulb in rats and mice. Localization of the factor in ensheathing cells, subsets of olfactory neuronal axons, and possibly maturing interneurons indicates a specific role of the factor in different processes of plasticity in the olfactory system. The findings provide a basis for further investigations into the functions of CNTF by analysis of possible changes in the olfactory sytem in CNTF−/− mice.

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REFERENCES

Asan E (1998) The catecholaminergic innervation of the rat amygdala. Adv Anat Embryol Cell Biol 142:1–121.

Asan E, Meyer-Stiegen S (1998) Localization of proteins associated with the junctional complex in the rat olfactory epithelium. Eur J Neurosci 10 Suppl 10360.

Bajetto A, Schettini G, Chimini G (1999) Nuclear localization of ciliary neurotrophic factor in glial cells. Brain Res 818:565–569.

Bajetto A, Barbero S, Bonavia R, Chimini G, Schettini G (2000) Immunofluorescence and biochemical techniques to detect nuclear localization of ciliary neurotrophic factor in glial cells. Brain Res Brain Res Protoc 3:273–281.

Barnett SC, Alexander CL, Iwashita Y, Gilson JM, Crowther J, Clark L, Dunn LT, Papanastassiou V, Kennedy PGE, Franklin RJM (2000) Identification of a human olfactory ensheathing cell that can effect transplant-mediated remyelination of demylinated CNS axons. Brain 123:1581–1588.

Bartolomei JC, Greer CA (2000) Olfactory ensheathing cells: bridging the gap in spinal cord injury. Neurosurgery 47:1057–1069.

Benson DL, Schnapp LM, Shapiro L, Huntley GW (2000) Making memories stick: cell-adhesion molecules in synaptic plasticity. Trends Cell Biol 10:473–482.

Berod A, Hartman BK, Pujol JF (1981) Importance of fixation in immunohistochemistry: use of formaldehyde solutions at variable pH for the localization of tyrosine hydroxylase. J Histochem Cytochem 29:844–850.

Bonfanti L, Theodosis DT (1994) Expression of polysialylated neural cell adhesion molecule by proliferating cells in the subependymal layer of the adult rat, in its rostral extension and in the olfactory bulb. Neuroscience 62:291–305.

Bonfanti L, Peretto P, Merighi A, Fasolo A (1997) Newly generated cells from the rostral migratory stream in the accessory olfactory bulb of the adult rat. Neuroscience 81:489–502.

Boruch AV, Conners JJ, Pipitone M, Deadwyler G, Storer PD, Devries GH, Jones KJ (2001) Neurotrophic and migratory properties of an olfactory ensheathing cell line. Glia 33:225–229.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Buckland ME, Cunningham AM (1999) Alterations in expression of the neurotrophic factors glial cell line-derived neurotrophic factor, ciliary neurotrophic factor and brain-derived neurotrophic factor, in the target-deprived olfactory neuroepithelium. Neuroscience 90:333–347.

Carlen M, Cassidy RM, Brismar H, Smith GA, Enquist LW, Frisen J (2002) Functional integration of adult-born neurons. Curr Biol 12: 606–608.

Chuah MI, Teague R (1999) Basic fibroblast growth factor in the primary olfactory pathway: mitogenic effect on ensheathing cells. Neuroscience 88:1043–1050.

Chuah MI, Cossins J, Woodhall E, Tennent R, Nash G, West AK (2000) Glial growth factor 2 induces proliferation and structural changes in ensheathing cells. Brain Res 857:265–274.

Dallner C, Woods AG, Deller T, Kirsch M, Hofmann HD (2002) CNTF and CNTF receptor alpha are constitutively expressed by astrocytes in the mouse brain. Glia 37:374–378.

Dobrea GM, Unnerstall JR, Rao MS (1992) The expression of CNTF message and immunoreactivity in the central and peripheral nervous system of the rat. Brain Res Dev Brain Res 66:209–219.

Emerich DF, Bruhn S, Chu Y, Kordower JH (1998) Cellular delivery of CNTF but not NT-4/5 prevents degeneration of striatal neurons in a rodent model of Huntington's disease. Cell Transplant 7:213–225.

Franklin RJ, Barnett SC (2000) Olfactory ensheathing cells and CNS regeneration: the sweet smell of success? Neuron 28:15–18.

Gall C, Berschauer R, Isackson P (1994) Seizures increase basic fibroblast growth factor mRNA in adult rat forebrain neurons and glia. Mol Brain Res 21:190–205.

Gheusi G, Cremer H, McLean H, Chazal G, Vincent JD, Lledo PM (2000) Importance of newly generated neurons in the adult olfactory bulb for odor discrimination. Proc Natl Acad Sci USA 97:1823–1828.

Giess R, Goetz R, Schrank B, Ochs G, Sendtner M, Toyka K (1998) Potential implications of a ciliary neurotrophic factor gene mutation in a German population of patients with motor neuron disease. Muscle Nerve 21:236–238.

Giess R, Maurer M, Linker R, Gold R, Warmuth-Metz M, Toyka KV, Sendtner M, Rieckmann P (2002) Association of a null mutation in the CNTF gene with early onset of multiple sclerosis. Arch Neurol 59:407–409.

Goodman MN, Silver J, Jacobberger JW (1993) Establishment and neurite outgrowth properties of neonatal and adult rat olfactory bulb glial cell lines. Brain Res 619:199–213.

Gudino-Cabrera C, Nieto-Sampedro M (2000) Schwann-like macroglia in adult rat brain. Glia 30:49–63.

Guthrie KM, Woods AG, Nguyen T, Gall CM (1997) Astroglial ciliary neurotrophic factor mRNA expression is increased in fields of axonal sprouting in deafferented hippocampus. J Comp Neurol 386: 137–148.

Henderson J, Seniuk N, Roder J (1994) Localization of CNTF immunoreacitvity to neurons and astroglia in the CNS. Mol Brain Res 22:151–165.

Imaizumi T, Lankford KL, Waxman SG, Greer CA, Kocsis JD (1998) Transplanted olfactory ensheathing cells remyelinate and enhance axonal conduction in the demyelinated dorsal columns of the rat spinal cord. J Neurosci 18:6176–6185.

Ip NY, Yancopoulos GD (1996) The neurotrophins and CNTF: two families of collaborative neurotrophic factors. Annu Rev Neurosci 19:491–515.

Joy A, Moffett J, Neary K, Mordechai E, Stachowiak EK, Coons S, Rankin-Shapiro J, Florkiewicz RZ, Stachowiak MK (1997) Nuclear accumulation of FGF-2 is associated with proliferation of human astrocytes and glioma cells. Oncogene 14:171–183.

Kafitz KW, Greer CA (1999) Olfactory ensheathing cells promote neurite extension from embryonic olfactory receptor cells in vitro. Glia 25:99–110.

Kalderon D, Roberts BL, Richardson WD, Smith AE (1984) A short amino acid sequence able to specify nuclear location. Cell 39:499–509.

Kott J, Westrum L, Raines E, Sasahara M, Ross R (1994) Olfactory ensheathing glia and platelet-derived growth factor B-chain reactivity in the transplanted rat olfactory bulb. Int J Dev Neurosci 12:315–323.

Lakatos A, Franklin RJ, Barnett SC (2000) Olfactory ensheathing cells and Schwann cells differ in their in vitro interactions with astrocytes. Glia 32:214–225.

Lee MY, Deller T, Kirsch M, Frotscher M, Hofmann HD (1997) Differential regulation of ciliary neurotrophic factor (CNTF) and CNTF receptor alpha expression in astrocytes and neurons of the fascia dentata after entorhinal cortex lesion. J Neurosci 17:1137–1146.

Levison SW, Jiang FJ, Stoltzfus OK, Ducceschi MH (2000) IL-6-type cytokines enhance epidermal growth factor-stimulated astrocyte proliferation. Glia 32:328–337.

Liposits Z, Phelix C, Paull WK (1986) A combined light and electron microscopic immunocytochemical method for the simultaneous localization of multiple tissue antigens. Histochemistry 85:95–106.

Lu J, Feron F, Ho SM, Mackay-Sim A, Waite PM (2001) Transplantation of nasal olfactory tissue promotes partial recovery in paraplegic adult rats. Brain Res 889:344–357.

Lu J, Feron F, Mackay-Sim A, Waite PM (2002) Olfactory ensheathing cells promote locomotor recovery after delayed transplantation into transected spinal cord. Brain 125:14–21.

Luskin MB (1993) Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. Neuron 11:173–189.

Mackay-Sim A, Chuah MI (2000) Neurotrophic factors in the primary olfactory pathway. Prog Neurobiol 62:527–559.

MacKenzie A, Wilson HL, Kiss-Toth E, Dower SK, North RA, Surprenant A (2001) Rapid secretion of interleukin-1beta by microvesicle shedding. Immunity 15:825–835.

Martinez-Marcos A, Ubeda-Banon I, Deng L, Halpern M (2000) Neurogenesis in the vomeronasal epithelium of adult rats: evidence for different mechanisms for growth and neuronal turnover. J Neurobiol 44:423–435.

Masu Y, Wolf E, Holtmann B, Sendtner M, Brem G, Thoenen H (1993) Disruption of the CNTF gene results in motor neuron degeneration. Nature 365:27–32.

Miragall F, Kadmon G, Husmann M, Schachner M (1988) Expression of cell adhesion molecules in the olfactory system of the adult mouse: presence of the embryonic form of N-CAM. Dev Biol 129: 516–531.

Mittoux V, Joseph JM, Conde F, Palfi S, Dautry C, Poyot T, Bloch J, Deglon N, Ouary S, Nimchinsky EA, Brouillet E, Hof PR, Peschanski M, Aebischer P, Hantraye P (2000) Restoration of cognitive and motor functions by ciliary neurotrophic factor in a primate model of Huntington's disease. Hum Gene Ther 11:1177–1187.

Moffett J, Kratz E, Florkiewicz R, Stachowiak MK (1996) Promoter regions involved in density-dependent regulation of basic fibroblast factor gene expression in human astrocytic cells. Proc Natl Acad Sci USA 93:2470–2475.

Moffett J, Kratz E, Myers J, Stachowiak EK, Florkiewicz RZ, Stachowiak MK (1998) Transcriptional regulation of fibroblast growth factor-2 expression in human astrocytes: implications for cell plasticity. Mol Biol Cell 9:2269–2285.

Peretto P, Merighi A, Fasolo A, Bonfanti L (1999) The subependymal layer in rodents: a site of structural plasticity and cell migration in the adult mammalian brain. Brain Res Bull 49:221–243.

Perez-Bouza A, Wigley CB, Nacimiento W, Noth J, Brook GA (1998) Spontaneous orientation of transplanted olfactory glia influences axonal regeneration. Neuroreport 9:2971–2975.

Raisman G (2000) Repair of corticospinal axons by transplantation of olfactory ensheathing cells. Novartis Found Symp 231:94–97.

Ramon-Cueto A, Valverde F (1995) Olfactory bulb ensheathing glia: a unique cell type with axonal growth-promoting properties. Glia 14: 163–173.

Ramon-Cueto A, Avila J (1998) Olfactory ensheathing glia: properties and function. Brain Res Bull 46:175–187.

Ramon-Cueto A, Cordero MI, Santos-Benito FF, Avila J (2000) Functional recovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia. Neuron 25:425–435.

Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17:208–212.

Saadat S, Sendtner M, Rohrer H (1989) Ciliary neurotrophic factor induces cholinergic differentiation of rat sympathetic in culture. J Cell Biol 108:1807–1816.

Sasahara A, Kott JN, Sasahara M, Raines EW, Ross R, Westrum LE (1992) Platelet-derived growth factor B-chain-like immunoreactivity in the developing and adult rat brain. Dev Brain Res 68:41–53.

Sendtner M, Carroll P, Holtmann B, Hughes RA, Thoenen H (1994) Ciliary neurotrophic factor. J Neurobiol 25:1436–1453.

Shapiro L, Colman DR (1999) The diversity of cadherins and implications for a synaptic adhesive code in the CNS. Neuron 23:427–430.

Sleeman MW, Anderson KD, Lambert PD, Yancopoulos GD, Wiegand SJ (2000) The ciliary neurotrophic factor and its receptor, CNTFR alpha. Pharm Acta Helv 74:265–272.

Smale KA, Doucelle R, Kawaja MD (1996) Implantation of olfactory ensheathing cells in the adult rat brain following fimbria-fornix transection. Exp Neurol 13:225–233.

Squinto SP, Aldrich TH, Lindsay RM, Morrissey DM, Panayotatos N, Bianco SM, Furth ME, Yancopoulos GD (1990) Identification of functional receptors for ciliary neurotrophic factor on neuronal cell lines and primary neurons. Neuron 6:757–766.

Stachowiak MK, Moffett J, Maher P, Tucholski J, Stachowiak EK (1997) Growth factor regulation of cell growth and proliferation in the nervous system: a new intracrine nuclear mechanism. Mol Neurobiol 15:257–283.

Stefanuto G, Cerrato M, Chiocchetti A, Tolosano E, Hirsch E, Cristofori R, Silengo L, Altruda F (1995) Analysis of regulatory regions of the ciliary neurotrophic factor gene in transgenic mice. Neuroreport 7:57–60.

Stöckli KA, Lillien LE, Naher-Noe M, Breitfeld G, Hughes RA, Raff MC, Thoenen H, Sendtner M (1991) Regional distribution, developmental changes, and cellular localization of CNTF-mRNA and protein in the rat brain. J Cell Biol 115:447–459.

Takahashi R, Yokoji H, Misawa H, Hayashi M, Hu J, Deguchi T (1994) A null mutation in the human CNTF gene is not causally related to neurological diseases. Nat Genet 7:79–84.

Thome J, Nara K, Foley P, Michel T, Gsell W, Retz W, Rosler M, Riederer P (1997) Ciliary neurotrophic factor (CNTF) genotypes: influence on choline acetyltransferase (ChAT) and acetylcholine esterase (AChE) activities and neurotrophin 3 (NT3) concentration in human post mortem brain tissue. J Brain Res 38:443–451.

Tisay KT, Key B (1999) The extracellular matrix modulates olfactory neurite outgrowth on ensheathing cells. J Neurosci 19:9890–9899.

Weisenhorn DM, Roback J, Young AN, Wainer BH (1999) Cellular aspects of trophic actions in the nervous system. Int Rev Cytol 189:177–265.

Wewetzer K, Grothe C, Claus P (2001) *In vitro* expression and regulation of ciliary neurotrophic factor and its alpha subunit in neonatal rat olfactory ensheathing cells. Neurosci Lett 306:165–168.

Winter CG, Saotome Y, Levison SW, Hirsh D (1995) A role for ciliary neurotrophic factor as an inducer of reactive gliosis, the glial response to central nervous system injury. Proc Natl Acad Sci USA 92:5865–5869.

Ye J-H, Houle JD (1997) Treatment of the chronically injured spinal cord with neurotrophic factors can promote axonal regeneration from supraspinal neurons. Exp Neurol 143:70–81.

Yoshihara Y, Mori K (1997) Basic principles and molecular mechanisms of olfactory axon pathfinding. Cell Tissue Res 290:457–463.

Zaborszky L, Heimer L (1989) Combinations of tracer techniques, especially HRP and PHA-L, with transmitter identification for correlated light and electron microscopic studies In: Neuroanatomical tract-tracing methods 2 (Heimer L, Zaborszky L, eds), pp. 49–96. New York: Plenum Press.

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