

Ciliary Neurotrophic Factor (CNTF) Enhances Myelin Formation: A Novel Role for CNTF and CNTF-Related Molecules

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In multiple sclerosis, myelin repair is generally insufficient despite the relative survival of oligodendrocytes within the plaques and the recruitment of oligodendrocyte precursors. Promoting remyelination appears to be a crucial therapeutic challenge. Using a newly developed enzymatic index of myelination, we screened different neurotrophic factors for their ability to enhance myelination. Neurotrophins [NGF, neurotrophin-3 (NT-3), NT-4/5, BDNF], glial cell line-derived neurotrophic factor (GDNF)-related factors (GDNF, neurturin), and growth factors such as PDGF-AA, FGF-2, and insulin did not increase myelinogenesis. In contrast, among factors belonging to the CNTF family, CNTF, leukemia inhibitory factor, cardiotrophin-1, and

oncostatin M induced a strong promyelinating effect. We provide evidence that CNTF acts on oligodendrocytes by favoring their final maturation, and that this effect is mediated through the 130 kDa glycoprotein receptor common to the CNTF family and transduced through the Janus kinase pathway. Our results demonstrate a novel role for neurotrophic factors of the CNTF family and raise the possibility that these factors might be of therapeutic interest to promote remyelination in multiple sclerosis.

Key words: oligodendrocytes; multiple sclerosis; myelination; remyelination; neurotrophins; growth factors; ciliary neurotrophic factor

In the CNS, oligodendrocytes synthesize large amounts of membranes that wrap around axons and compact to form myelin. Myelinated axons have the ability to elicit rapid, saltatory conduction of action potentials. In demyelinating diseases such as multiple sclerosis (MS), the destruction of myelin sheaths may result in a slowing down or even a complete block of electrical conduction. In addition, there has been growing evidence that demyelinated axons are more sensitive to severe injury (Ferguson et al., 1997; Trapp et al., 1998). Spontaneous remyelination occurs in MS, but the extent of myelin repair remains insufficient to prevent the progression of disability. Failure to remyelinate may take place despite the survival of oligodendrocytes (Lucchinetti et al., 1996, 1999) and/or recruitment of oligodendrocyte precursor cells (OPCs) around and within MS lesions (Wolswijk, 1998; Chang et al., 2000; Dawson et al., 2000; Maeda et al., 2001). This suggests that failure of remyelination is not the exclusive consequence of the death of oligodendrocytes, but may also be the result of an incapability of OPCs to mature into myelin-forming cells or of differentiated oligodendrocytes to achieve their normal function (i.e., synthesize myelin membrane). The persistence in MS plaques of quiescent oligodendrocytes and OPCs could be related to the presence of local environmental cues that inhibit their final maturation or to the depletion of factors that normally promote myelination.

Although there is growing knowledge about the various factors involved in the induction and specification of oligodendrocyte lineage, as well as about proliferation, survival, control of the cell cycle, and differentiation of oligodendrocytes, little is known about the molecular control of the myelination process itself. During development in the brain, myelination follows a caudorostral gradient, suggesting the existence of a spatiotemporal control. Myelination requires a tightly regulated balance between the disappearance of inhibitory signals, one of them being the downregulation of the polysialylated neural cell adhesion molecule from the axonal surface (Charles et al., 2000), and the induction of positive signals, some of which are mediated by the neuronal electrical activity (Demerens et al., 1996). Because neurotrophic factors have been shown to play a role in the proliferation and survival of OPCs, we questioned whether these factors could also interact with oligodendroglial cells at later stages during their development to promote their maturation into myelin-forming cells. Here we show that neurotrophins or glial cell line-derived neurotrophic factor (GDNF)-related factors had no promyelinating activity, whereas members of the ciliary neurotrophic factor (CNTF) family strongly promote myelin formation by activating the 130 kDa glycoprotein Janus kinase (gp130-JAK) pathway.

MATERIALS AND METHODS

Animals. The 1900bp-MBP lacZ transgenic mouse was kindly supplied by Dr. R. Lazzarini (Gow et al., 1992). The 1900bp-MBP lacZ transgene consists of the *Escherichia coli* lacZ reporter gene flanked upstream by a DNA fragment that extends from position +36 to position -1907 of the murine MBP gene promoter region. We have shown previously that this proximal portion of the MBP promoter region is turned on after MBP expression and only at the time of myelination (Stankoff et al., 1996). The *plp-sh ble-lacZ* (*plp-lacZ*) transgenic mouse has been described previously (Spassky et al., 1998). In this transgenic line the transgene is detected in

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early oligodendrocyte progenitors but is highly upregulated at the time of myelination. For myelinating cultures, animals were obtained by crossing homozygous transgenic mice with nontransgenic OF1 (Oncins France, Strain 1) mice (Iffacredo, L'Arbresle, France).

Antibodies and reagents. Mouse monoclonal O4 (IgM) (Sommer and Schachner, 1981) and anti-galactosylceramide (GalC) antibodies (R-mAb, IgG₃) (Ranscht et al., 1982) were diluted in 10% fetal calf serum (Eurobio, Les Ulis, France), 1:5 and 1:30, respectively. Mouse monoclonal anti-myelin basic protein (MBP) (IgG₁, culture supernatant from clone M-010h; Euromedex, Souffelweysheim, France) and anti-myelin oligodendrocyte glycoprotein (MOG) (IgG₁, culture supernatant from clone 8-18C5) (Linnington et al., 1984) antibodies were diluted 1:100 and 1:10, respectively, in 0.2% gelatin and 0.2% Triton X-100 PBS. Texas Red- and fluorescein-conjugated sheep antibodies against mouse IgG₁ and IgG₃ and fluorescein-conjugated goat antibodies against mouse IgM (Southern Biotechnology, Birmingham, AL) were used diluted 1:100. Biotin-conjugated goat antibody against mouse IgG₁ (Amersham Biosciences, Arlington Heights, IL) was used at a dilution of 1:100.

Mouse recombinant interleukin-6 (IL-6) and nerve growth factor (NGF), rat recombinant CNTF, human recombinant brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4, glial cell-derived neurotrophic factor (GDNF), neurturin (NTU), cardiotrophin-1 (CT-1), leukemia inhibitory factor (LIF), and fibroblast growth factor 2 (FGF-2) were obtained from Alomone Labs (Jerusalem, Israel). Human recombinant IL-6, IL-11, oncostatin M (OsM), and human recombinant soluble α -chain of IL-6 receptor were supplied by Chemicon (Temecula, CA). Human recombinant platelet-derived growth factor (PDGF-AA) was purchased from Upstate Biotechnology (Lake Placid, NY).

Myelinating cultures. Cultures were performed either on poly-L-lysine-coated 14 mm glass coverslips (OSI, Maurepas, France) or directly on poly-L-lysine-coated 24-well plastic plates. Forebrains were removed from 15-d-old mouse fetuses heterozygous either for the *MBP-lacZ* transgene or for the *plp-sh ble-lacZ* transgene. They were dissociated mechanically and by enzymatic digestion with 0.025% trypsin (Biological Industries, Kibbutz Beit Haemek, Israel) for 15 min at 37°C. After washing, the pellet was passed gently through a nylon mesh (63 μ m) and then resuspended in DMEM (Seromed, Noisy le Grand, France) containing 10% FCS (Eurobio, Les Ulis, France). A total of 5×10^4 cells per well were plated and seeded in DMEM containing 10% FCS to facilitate attachment for 30 min; then 500 μ l of culture medium was added to each well. Standard culture medium consisted of Bottenstein and Sato (BS) medium (Bottenstein et al., 1979) supplemented with 0.5% FCS and 1% penicillin–streptomycin (Biological Industries). During the first week of culture, 10 ng/ml recombinant PDGF-AA (Upstate Biotechnology) was added. Neurotrophic factors were added between 11 and 25 d *in vitro* (DIV), and FCS was removed. As described previously (Demerens et al., 1996), myelinated segments were identified as bright double MOG⁺ lines. For direct quantification of myelin formation, the total number of myelinated internodes for each 14 mm coverslip was counted and compared with control cultures. Controls were sister cultures from the same experiment.

Immunolabeling. Coverslips were fixed with 4% paraformaldehyde (PFA) in PBS at room temperature for 15 min and then saturated with DMEM containing 10% FCS and 50% sheep serum for 20 min. Primary antibodies were diluted either in DMEM containing 10% FCS (O4, GalC) or in 0.2% Triton X-100 and 2 gm/l gelatin in PBS (MBP, MOG) and incubated for 30 min at room temperature. After washing, cultures were incubated with the secondary fluorochrome-conjugated antibody for 30 min and coverslips were mounted in fluoromount G (Southern Biotechnology Associates Inc., Birmingham, AL) to prevent fading of fluorescence. For immunoperoxidase staining, endogenous peroxidase was inhibited by immersion of cultures in 1.5% H₂O₂ in PBS for 15 min at room temperature. The coverslips were then incubated with the MBP antibody diluted in 0.2% Triton X-100 and 2 gm/l gelatin in PBS at 4°C overnight before incubation with the biotin-conjugated secondary antibody for 1 hr at room temperature and then with the Vectastain-Elite-ABC reagent (Vector Laboratories, Burlingame, CA). After two washes (10 min each) in 0.1 M Tris-HCl, pH 7.6, peroxidase activity was revealed using 3,3'-diaminobenzidine tetrahydrochloride (Dakopatts, Glostrup, Denmark) as a chromogen at a concentration of 1 mg/ml in 0.1 M Tris-HCl, pH 7.6. After washes in PBS, coverslips were mounted in fluoromount G.

5-bromo-4-chloro-3-indolyl- β -D-galactoside staining. Cultures were fixed in 4% PFA for 2 min at room temperature and rinsed twice in PBS before incubation for 1–3 hr at 37°C in the staining solution consisting of

(in mM): 2 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) (United States Biochemicals, Cleveland, OH), 20 potassium ferrocyanide, 20 potassium ferricyanide, and 2 MgCl₂ in PBS. After washing in PBS, cells were postfixed in 4% PFA for 15 min at room temperature.

Enzymatic assay of β -galactosidase activity. β -galactosidase activity was assayed in each culture well using the galactolight-plus kit (Tropix Inc., Bedford, MA). Cells were lysed in a solution containing 100 mM potassium phosphate, 0.2% Triton X-100, and 0.5 mM dithiothreitol. Samples were centrifuged for 5 min to pellet debris, and supernatants were transferred into fresh microfuge tubes and frozen at -80°C until used. For β -galactosidase detection, 20 μ l of the extracts was diluted in 200 μ l of the reaction buffer (galacton-plus substrate diluted 1:100 in reaction buffer diluent) and incubated in the dark at room temperature for 1 hr. The Light Emission Accelerator (300 μ l/sample; Tropix) was then injected in the same consistent time frame that the reaction buffer was added and the luminescence was quantitated in a Beckman (Fullerton, CA) scintillation counter using the single monitor software. Results were expressed in counts per minute per nanogram of protein. Protein concentrations were determined according to Bradford (1976), using bovine serum albumin as a standard.

RESULTS

In myelinating cocultures derived from 1900bp-MBP *lacZ* transgenic brains, assay of β -galactosidase enzymatic activity provides a reliable index of myelination

In cocultures derived from embryonic 1900bp-MBP *lacZ* cerebral hemispheres, oligodendroglial differentiation and myelination occurred with the same timing as described previously for similar cultures derived from wild-type animals (Lubetzki et al., 1993; Demerens et al., 1996). After 11–12 DIV, the oligodendroglial population consisted of O4⁺ preoligodendrocytes (Sommer and Schachner, 1981) and GalC-expressing immature oligodendrocytes labeled with the R-mAb (Ranscht et al., 1982). The first MBP-expressing cells were detected at 12–14 DIV. More mature MOG⁺ oligodendrocytes, characterized by their pauci-branched arborization, started to be observed at 15 DIV. The drastic change in morphology observed between 12 and 15 DIV corresponds to the transition between a mature non-myelin-forming cell (MOG[−]) (Fig. 1A,C) and a myelinating phenotype (MOG⁺) (Fig. 1B) (Solly et al., 1996). Combination of X-gal staining, to detect β -galactosidase enzymatic activity, and anti-MBP immunolabeling demonstrated that, in myelinating cocultures derived from 1900bp-MBP *lacZ* animals, transgene expression was restricted to mature pauci-branched oligodendrocytes, either myelinating (Fig. 1E) or pseudo-myelinating (Fig. 1D). Pseudo-myelinating oligodendrocytes had not established contact with axons but are MOG⁺ cells, which have formed whorls of poorly compacted membranes at the tip of their processes (Fig. 1B,D), as shown previously by electron microscopy (Lubetzki et al., 1993). In addition, all β -galactosidase-positive cells were also MOG⁺ (data not shown). In contrast, MOG[−] multibranching non-myelin-forming cells (Fig. 1A,B) were never stained with the X-gal substrate, and thus did not express detectable levels of β -galactosidase (Fig. 1A–C).

β -galactosidase enzymatic activity was assayed and compared with the stage of differentiation of the oligodendroglial population (Fig. 2). As expected, no β -galactosidase enzymatic activity was detected before myelin deposition and/or MOG expression: β -galactosidase activity was first detected at 15 DIV and then increased threefold and eightfold at 19 and 25 DIV, respectively. This increase paralleled myelin formation in the cultures, as evaluated by the increase in the number of myelinated internodes (Fig. 2A). During the same period, the ratio of GalC⁺ oligodendrocytes double-labeled with anti-MOG mAb increased from 15

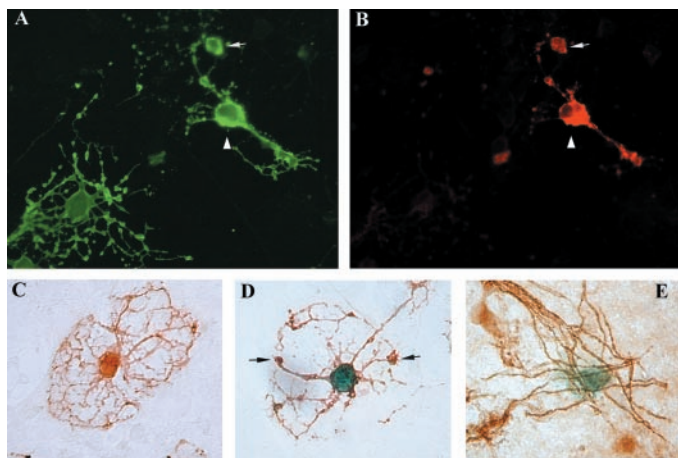


Figure 1. In cultures derived from *1900bp-MBP lacZ* fetuses, β -galactosidase expression is restricted to myelinating oligodendrocytes. Dissociated cultures from E15 *1900bp-MBP lacZ* mouse brain cultured for 3 weeks were doubly stained with anti-GalC (A) and anti-MOG (B) antibodies or with anti-MBP antibodies and X-gal substrate to reveal β -galactosidase enzymatic activity (C, D, E). A, B, The same field was photographed with fluorescein (A) and rhodamine (B) optics. In A, two GalC⁺ cells are seen (green): the cell in the bottom left has the typical multibranch morphology of a mature non-myelin-forming oligodendrocyte. This cell is MOG[−] (B). In contrast, in the top right, another GalC⁺ cell is also MOG⁺ (red). This GalC⁺/MOG⁺ cell (arrowhead points to the cell body) is identified as a pseudo-myelinating oligodendrocyte based on its poorly branched morphology and the presence of whorls of myelin-like figures at the tips of its processes (arrows). C, A mature non-myelin-forming oligodendrocyte with the typical “sun-like” morphology is MBP[−] (brown) but X-gal-negative. D, A pseudo-myelinating oligodendrocyte with characteristic whorls of myelin-like figures (arrows) is MBP⁺ (brown) and X-gal-positive (blue staining of the cell body). E, A typical field of fibers myelinated by a single oligodendrocyte. This myelinating oligodendrocyte is MBP⁺/X-gal⁺. Note that the myelinated internodes are strongly MBP⁺, whereas the X-gal⁺ cell body appears MBP[−] because in myelinating oligodendrocytes most of the MBP migrates out of the cell body and MBP immunoreactivity is mostly confined to the myelin sheath. Scale bars, 10 μ m.

to 39%, whereas the number of O4⁺ cells remained relatively stable. Because GalC⁺ and MOG⁺ cells still retain the expression of O4-recognized antigen (Bansal et al., 1989), the stability of the O4⁺ population suggested that after 11 DIV few if any, new oligodendrocyte precursor cells were generated under our culture conditions (Fig. 2B). These data demonstrated that in the *1900bp-MBP lacZ* transgenic line, the increase in the level of β -galactosidase between 15 and 25 DIV in the absence of growth factor specifically reflects the increase in myelin formation and not the generation of new oligodendrocytes. Therefore, quantification of β -galactosidase enzymatic activity could be used as a reliable index of myelination in control situations.

Effect of different neurotrophic and growth factors on myelination

We used this assay to screen for chemokines or cytokines that could play a role in myelin formation. Based on the family of receptors they activate, the factors investigated were classified into four groups: (1) neurotrophins (NGF, BDNF, NT-3, and NT-4/5) acting through the Trk family of receptors; (2) GDNF and NTU, which are growth factors acting through Ret receptors; (3) PDGF-AA, FGF-2, or insulin, which mediate their action by binding to tyrosine kinase receptors; and finally (4) members of the CNTF family of ligands, which interact with gp130-containing receptors. The factors to be tested were added to the culture

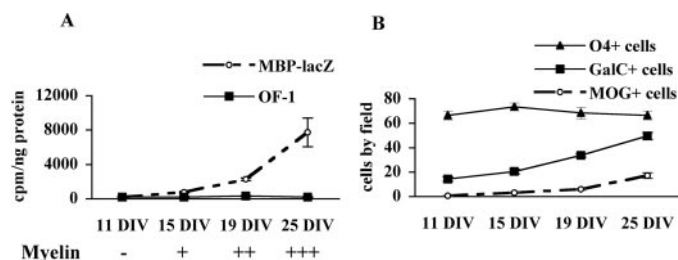


Figure 2. Assay of β -galactosidase enzymatic activity in myelinating cultures derived from *1900bp-MBP lacZ* provides an index of myelination. A, β -galactosidase activity (expressed in counts per minute per nanogram of protein) was assayed as a function of time *in vitro*. In cultures derived from *1900bp-MBP lacZ*, β -galactosidase activity started to be detected at 15 DIV, whereas no activity was detectable in cultures derived from nontransgenic OF1 animals. The intensity of myelination in sister cultures was evaluated by counting the number of myelinated MBP positive internodes and is indicated by + (onset), ++ (moderate), and +++ (maximum). B, Sister cultures were also immunostained with O4, and either anti-GalC or anti-MOG mAbs. Note that the number of O4⁺ cells remained constant between 11 and 25 DIV, whereas the number of GalC⁺ cells increased during the same period. MOG⁺ cells were not detected before 15 DIV, and the increase in their number paralleled the increase in β -galactosidase activity (A). Results are expressed as the number of positive cells per field (objective, 40 \times) (means \pm SEM of cell count from 6 fields per culture, with 4–6 cultures per experiment).

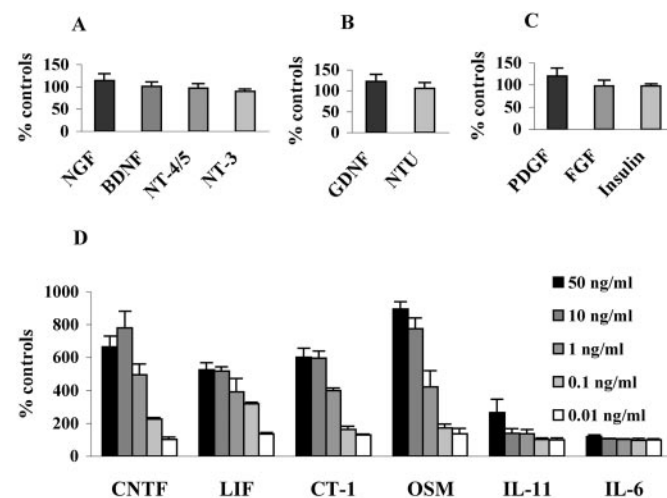


Figure 3. Effect on myelination of different neurotrophic and growth factors. Myelinating cultures derived from *1900bp-MBP lacZ* embryos were treated between 11 and 25 DIV; β -galactosidase activity was assayed at 25 DIV. A–C, Members of the neurotrophin (A) and GDNF (B) families and growth factors (C) were used at a concentration of 10 ng/ml, except for insulin, which was used at 100 μ g/ml. D, Members of the CNTF family were tested at concentrations varying between 0.01 and 50 ng/ml. For each culture well, myelin formation was assessed by the quantification of the β -galactosidase level normalized to protein level (expressed as counts per minute per nanogram of protein). Results are expressed as the percentage of mean control values. A–C, Results are means \pm SEM of three independent experiments with four to six cultures per experiment. D, Results are the means \pm SEM of six cultures from one representative experiment.

medium of myelinating cocultures, derived from embryonic day 15 (E15) *1900bp-MBP lacZ* transgenic animals between 11 and 25 DIV, and myelin formation was quantified by assaying β -galactosidase enzymatic activity at 25 DIV. No promyelinating effect was observed when neurotrophins (10 ng/ml) were added to the cultures (Fig. 3A). Neurotrophins were also used at concentrations varying between 0.1 and 50 ng/ml, without any effect

(data not shown). Similarly, GDNF, NTU (Fig. 3B), PDGF-AA, FGF-2 (each at 10 ng/ml), and insulin (100 μ g/ml) (Fig. 3C) did not promote myelin formation. In contrast, CNTF dramatically increased myelin formation: the mean \pm SEM β -galactosidase activity was 5.4 ± 0.9 -fold higher in cultures treated with CNTF (10 ng/ml) than in untreated control cultures. The treatment of cultures with concentrations of CNTF varying between 0.01 and 50 ng/ml showed a clear dose response (Fig. 3D). The CNTF-related factors LIF, CT-1, OsM, IL-6, and IL-11 were also tested. LIF, CT-1, and OsM had a promyelinating effect of the same amplitude as CNTF. When used at 10 ng/ml each, the increase in myelin formation was 5.9 ± 0.9 -fold for CT-1, 4.6 ± 0.6 -fold for LIF, and 4.8 ± 0.3 -fold for OsM. This effect was clearly dose dependent (Fig. 3D). We did not observe any additive or synergistic action when these factors were added together (data not shown); addition of either neurotrophins or GDNF to CNTF-treated cultures did not potentiate the promyelinating effect of CNTF (data not shown). In contrast, IL-11 (2.3 ± 0.3 -fold increase) or IL-6 (no increase) had little or no effect on myelination (Fig. 3D). The lack of effect of IL-6 was not attributable to species specificity, because we evaluated both human- and mouse-derived IL-6 with the same negative result.

Promyelinating effect of CNTF is mediated by favoring oligodendrocyte maturation

To determine whether the promyelinating effect of CNTF was related to an effect on oligodendrocyte maturation, we evaluated the proportion of GalC⁺ oligodendrocytes coexpressing the maturation marker MOG. Compared with controls, treatment with CNTF resulted in a 1.9-fold increase in the percentage of GalC⁺/MOG⁺ mature oligodendrocytes (Fig. 4A). In addition, in separate experiments, the percentage of MOG⁺ cells among total O4⁺ oligodendrocytes was increased 1.8-fold in CNTF-treated cultures ($36.6 \pm 4.8\%$ vs $66.9 \pm 0.8\%$ in control and treated cultures, respectively). The promyelinating effect observed with CNTF was not attributable to a selective activation of the 1900bp-MBP promoter, because a 3.7 ± 0.3 -fold increase in β -galactosidase activity was observed after the addition of CNTF to cultures derived from *plp-lacZ* mice, another transgenic line, in which the *lacZ* transgene is under the control of the proteolipid protein (PLP) promoter (Spassky et al., 1998) (Fig. 4B). Finally, the promyelinating effect of CNTF detected by β -galactosidase assay was then confirmed by direct quantification of the extent of axon wrapping. In these latter experiments, an increase in the number of myelinated internodes was indeed observed for concentrations of CNTF of 0.1 and 1 ng/ml (Fig. 4C). Interestingly, the effect was less pronounced for higher concentrations of CNTF, suggesting that a strong upregulation of myelin gene transcription could compromise myelin formation or maintenance.

Cytokines activating the gp130/Janus kinase pathway stimulate CNS myelination

CNTF, LIF, OsM, and CT-1 use receptors containing LIF receptor (LIFR)/gp130 heterodimers, whereas IL-6 and IL-11 have been shown to act through gp130 homodimer-containing receptors. To demonstrate unambiguously that a promyelinating action could be obtained independently of the LIFR unit, we conducted additional experiments in which IL-6 and its soluble α -subunit receptor were added together. Whereas neither IL-6 nor the soluble α subunit alone induced any effect on myelin formation, the simultaneous addition of IL-6 and soluble α -subunit receptor caused a fivefold increase in β -galactosidase activity, but only

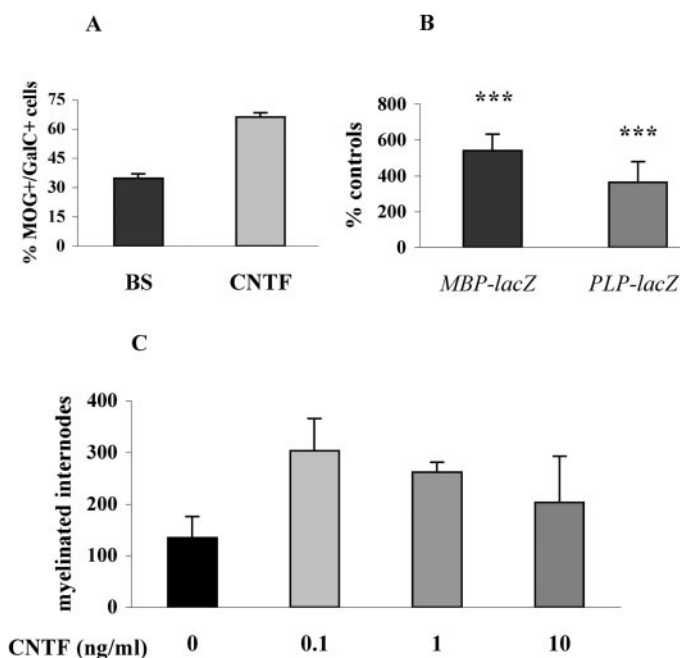


Figure 4. The promyelinating effect of CNTF is mediated by enhancing oligodendrocyte maturation. Myelinating cultures derived from 1900bp-MBP *lacZ* (A–C) or *plp-lacZ* (PLP-*lacZ*) embryos (B) were treated with CNTF and analyzed at 25 DIV for MOG expression (A), β -galactosidase activity (B), or axon wrapping (C). A, Dissociated cultures were doubly stained with anti-GalC and MOG mAb, and the percentage of GalC⁺ cells expressing MOG was quantified in CNTF-treated (10 ng/ml, 11–25 DIV) and control cultures. Results are expressed as the means \pm SEM of one representative experiment (determination from 6 different culture wells per condition). BS, Bottenstein and Sato medium. B, β -galactosidase activity, expressed as a percentage of control untreated cultures, was assayed in CNTF-treated (11–25 DIV) cultures derived from either 1900bp-MBP *lacZ* or *plp-lacZ* transgenic embryos. Results are the means \pm SEM of three independent experiments representing four to six cultures per experiment. *** p < 0.001; Student's t test. C, The number of myelinated internodes was counted in control and CNTF-treated cultures. Results are expressed as means \pm SEM (determination of 5 different culture wells per condition).

when IL-6 was used at a concentration of 50 ng/ml, and this activity was no longer detected at 10 ng/ml (Fig. 5A).

To determine whether the promyelinating effect of CNTF was mediated through the JAK, we attempted to block this transduction pathway using the JAK inhibitor AG490 (Meydan et al., 1996). Simultaneous addition of increasing concentrations of AG490 to CNTF (10 ng/ml) resulted in a dose-dependent inhibition of the promyelinating effect of CNTF, whereas AG490 alone had no influence on β -galactosidase activity (Fig. 5B).

DISCUSSION

In the 1900bp-MBP *lacZ* transgenic, assay of β -galactosidase enzymatic activity is a reliable and simple alternative to quantify myelin formation

In neuron–oligodendrocyte coculture, myelination was previously quantified by counting the number of either myelinated internodes or myelinating and pseudo-myelinating cells (Lubetzki et al., 1993; Demerens et al., 1996, 1999; Charles et al., 2000). However, this system of quantification is not suitable to screen for a large number of molecules influencing myelination. In the 1900bp-MBP *lacZ* transgenic mouse, we have shown previously that expression of the reporter transgene is turned on

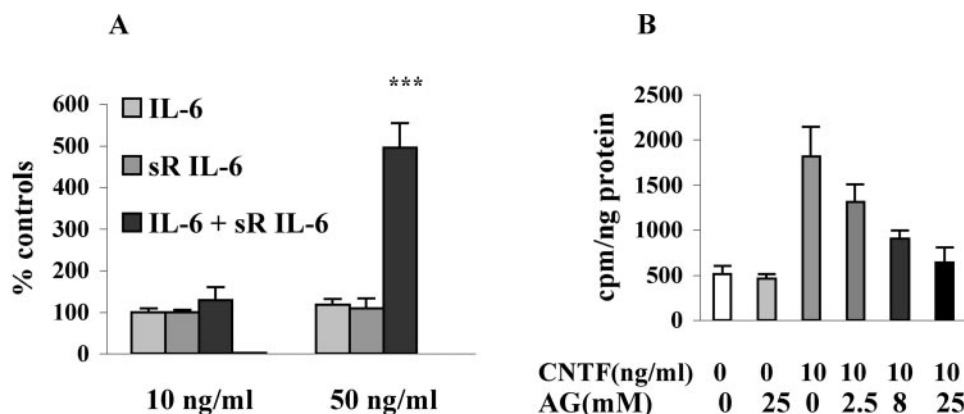


Figure 5. The promyelinating effect of CNTF is signaled by gp130-containing receptors. *A*, Cultures were treated with IL-6, the IL-6-soluble receptor α subunit (sR-IL-6), or IL-6 + sR-IL-6 at either 10 or 50 ng/ml each. Results are expressed as a percentage of control β -galactosidase levels (means \pm SEM of 3 independent experiments with 4–6 different cultures per experiment). *B*, CNTF (10 ng/ml) and JAK inhibitor AG490 (AG) were added daily into the culture medium between 14 and 18 DIV, and β -galactosidase was assayed at 19 DIV. β -galactosidase levels are expressed as means \pm SEM (6 different culture wells for each condition) of one representative experiment. *** p < 0.001; Student's t test.

after MBP expression and only when oligodendrocytes start to myelinate (Stankoff et al., 1996). Here we show that in myelinating cultures derived from 1900bp-MBP *lacZ* embryos, expression of the transgene is restricted to myelinating and pseudo-myelinating oligodendrocytes (Fig. 1). Pseudo-myelinating oligodendrocytes are mature oligodendrocytes that have not established an axonal contact *in vitro*. Our observation that the reporter gene is expressed by both myelinating and pseudo-myelinating cells makes it a powerful tool to screen for molecules that favor the final maturation of oligodendrocytes. Because the assay of β -galactosidase is easy and very sensitive, quantification of this enzymatic activity in myelinating cocultures derived from 1900bp-MBP *lacZ* transgenic mice provides a simple and reliable index of myelin synthesis. Using this index, we provide evidence that only CNTF-related factors induce a promyelinating effect.

CNTF-induced increase in myelination is not mediated by an increase in proliferation or survival of oligodendrocyte precursor cells

Because CNTF has been shown to increase the proliferation (Barres et al., 1996) and survival (Barres et al., 1993; Louis et al., 1993; Mayer et al., 1994) of oligodendrocyte precursors, the observed effect on myelination could be the consequence of a higher number of oligodendrocytes in the cultures. This is very unlikely for several reasons. First, we have evaluated bromodeoxyuridine incorporation in glial cultures derived from newborn animals and found no variation in oligodendrocyte precursor proliferation in the presence or absence of CNTF (B. Stankoff, unpublished results). Second, other growth factors with well documented mitogenic properties on oligodendrocyte precursors, such as PDGF-AA (Richardson et al., 1988), FGF-2 (Bogler et al., 1990), and NT-3 (Barres et al., 1994; Cohen et al., 1996), had no effect on the β -galactosidase level.

It has also been reported that CNTF promotes the survival of newly differentiated oligodendrocytes in culture. In these studies, oligodendrocyte survival has been evaluated either in low-density cultures (Barres et al., 1993) or in cultures grown in a poor minimal medium (Mayer et al., 1994) or against tumor necrosis factor α -induced toxicity (Louis et al., 1993), whereas in our experiments, cultures were at high density and grown in B-S medium containing survival factors such as PDGF-AA (during the first week *in vitro*) and high concentrations of insulin. The stability of the O4⁺ population of cells between 11 and 25 DIV indicates that a significant number of deaths of either oligodendrocytes or oligodendrocyte precursors did not occur during this period. Moreover, insulin alone, which is also a survival factor for oligodendrocytes in low-density cultures (Barres et al., 1993), had

no effect on β -galactosidase levels, and addition of insulin to CNTF-treated cultures did not increase myelination (data not shown).

Together, these data suggest that the increase in myelin formation induced by CNTF and CNTF-related factors is not the result of an increase in oligodendrocyte proliferation and survival, but relates to a specific effect on myelin synthesis. Furthermore, our observation that CNTF enhances the final maturation of oligodendrocytes by increasing the proportion of MOG⁺ myelinating oligodendrocytes as well as the increase in β -galactosidase activity measured in cultures derived from *plp-lacZ* transgenic mice are additional arguments in support of an effect of CNTF and CNTF-related molecules on myelin formation. In this respect, because the increase in MOG⁺ cells is smaller than the increase in β -galactosidase expression, it is likely that in addition to an effect on oligodendrocyte maturation, CNTF could also increase myelin synthesis per oligodendrocyte. A similar increase was also observed in the number of myelinated internodes, confirming the effect on myelin formation per se. However, the lower magnitude of this effect could be attributable to the limited number of qualified axons permissive to myelination (Charles et al., 2000). Together, our results provide evidence of a new role for CNTF and CNTF-related molecules. It is possible that CNTF acts directly on oligodendrocytes. Alternatively, because our cultures contain astrocytes in addition to neurons and oligodendrocytes, we cannot exclude the possibility that the reported effect on myelin formation is indirect, via astrocytes or neurons.

Promyelinating effect of CNTF is mediated by the LIFR β /gp130 complex

The promyelinating effect induced by CNTF and CNTF-related factors, such as LIF, CT-1, OsM, was of the same order of magnitude with no additive or synergistic action. This functional overlap between related cytokines suggests that their mechanism of action on myelination might involve a similar transducing pathway. These cytokines use receptors containing the same signal transducer gp130, which forms a heterodimer with the other related partner, LIFR β (Kishimoto, 1994; Stahl and Yancopoulos, 1994; Turnley and Bartlett, 2000). LIF binds with high affinity to the LIFR β /gp130 complex, whereas for CNTF, CT-1, and OsM, ligand specificity is conferred by a third α subunit, which forms a complex with the LIFR β /gp130 heterodimer. In contrast, neither IL-6 nor IL-11 acts through the formation of an LIFR β /gp130 heterodimer. Signaling of IL-6 requires binding to the IL-6 α -subunit receptor and homodimerization of gp130. For IL-11, two controversial modes of action have been proposed: formation of either a gp130 homodimer or a heterodimer of

gp130 with an as yet unidentified IL-11-specific subunit (Yin et al., 1993; Neddermann et al., 1996). Because IL-6 is inducing a promyelinating effect when added with its soluble α -subunit receptor, this suggests that dimerization of gp130 is sufficient to mediate this effect. However, to enhance myelin formation, IL-6 had to be used at a concentration at least fivefold higher than for CNTF, LIF, CT-1, or OsM. Therefore, it is likely that under physiological conditions, the promyelinating properties of CNTF-related factors use the formation of LIFR β /gp130 heterodimer.

Ligand binding followed by receptor complexing activates JAK (Stahl and Yancopoulos, 1994). The inhibition observed with the JAK inhibitor AG490 suggests that the CNTF promyelinating effect is signaled through the JAK pathway. Activation of JAK leads to docking of Src homology 2 domains of a variety of proteins, such as signal transducer and activator of transcription (STAT) proteins (Stahl and Yancopoulos, 1994; Segal and Greenberg, 1996). Activated STAT molecules dimerize and translocate to the nucleus, where they induce the expression of a variety of genes. Several JAK (JAK1, JAK2, Tyk2) and at least six different STAT proteins have been described. In addition to the JAK/STAT pathway, binding of CNTF-related factors can also activate the Ras mitogen-activated protein (MAP) kinase (Stahl et al., 1995; Giordano et al., 1997); in some cell types, signaling through phosphatidylinositol 3 (PI3) kinase has also been described previously (Oh et al., 1998). The different combination of JAK/STAT proteins and the alternative activation of MAP or PI3 kinases might explain the different types of responses induced in cells of the oligodendroglial lineage, depending on their developmental stages. For instance, because PDGF-AA and FGF-2 have been described as potent mitogens for oligodendrocyte precursors, and because signaling of these growth factors is mediated through the MAP kinase pathway, it can be postulated that CNTF-induced proliferation of oligodendrocyte precursors is also signaled by MAP kinase. PDGF-AA and CNTF also enhance oligodendrocyte precursor survival, and it has been shown that this survival effect is mediated by rapid tyrosine phosphorylation of JAK1, JAK2, STAT1 α/β , and STAT3 (Dell'Albani et al., 1998). Therefore, it is tempting to speculate that, later during development, binding of CNTF to mature oligodendrocytes activates a different combination of JAK and STAT proteins, which in turn induce, or enhance, the coordinate expression of the subset of myelin-specific genes necessary to synthesize a quantity of membrane sufficient to form the myelin sheath.

Does CNTF favor myelination *in vivo*?

In addition to their effect on neurons, CNTF-related factors have been shown to enhance proliferation, survival, and differentiation of oligodendrocyte precursor cells (Barres et al., 1993; Louis et al., 1993; Mayer et al., 1994; Barres et al., 1996; Vos et al., 1996; Marmur et al., 1998). However, to our knowledge, this is the first time that CNTF-related factors have been shown to enhance myelination by acting on the last stages of oligodendrocyte maturation. Interestingly, in the rat optic nerve, it has been shown that astrocytes start to synthesize CNTF at the end of the first postnatal week (Stockli et al., 1991; Dobrea et al., 1992), which corresponds to the onset of myelination (Colello et al., 1995). This temporal concordance supports a physiological role of CNTF on myelin formation. Nevertheless, in CNTF-deficient mice both oligodendrocyte number and myelination attain wild-type values (Barres et al., 1993), presumably because of compensation by either the other gp130-stimulating cytokines or by the cytokine-like factor-1/cardiotrophin-like cytokine complex, which

is the second ligand for the CNTF receptor (Elson et al., 2000). However, mice deficient for one of the main signaling elements involved in CNTF-related cytokine function, such as CNTF- α receptor, gp130, LIFR β , or JAKs, die during development or perinatally (DeChiara et al., 1995; Li et al., 1995; Ware et al., 1995; Parganas et al., 1998; Rodig et al., 1998). Therefore, these mutants have failed to provide any models to investigate the role of the CNTF signaling pathway in myelinogenesis. However, the promyelinating effect of CNTF-related factors and the observation that they could also promote the differentiation of postnatal brain precursor cells in oligodendrocytes (Marmur et al., 1998) could be of therapeutic value in MS.

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