Regulation of Gliogenesis in the Central Nervous System by the JAK-STAT Signaling Pathway

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A mechanism by which members of the ciliary neurotrophic factor (CNTF)–leukemia inhibitory factor cytokine family regulate gliogenesis in the developing mammalian central nervous system was characterized. Activation of the CNTF receptor promoted differentiation of cerebral cortical precursor cells into astrocytes and inhibited differentiation of cortical precursors along a neuronal lineage. Although CNTF stimulated both the Janus kinase–signal transducer and activator of transcription (JAK-STAT) and Ras–mitogen-activated protein kinase signaling pathways in cortical precursor cells, the JAK-STAT signaling pathway selectively enhanced differentiation of these precursors along a glial lineage. These findings suggest that cytokine activation of the JAK-STAT signaling pathway may be a mechanism by which cell fate is controlled during mammalian development.

The cells of the central nervous system (CNS) are thought to arise from multipotential precursor cells whose developmental potential becomes progressively restricted (*1*–*8*). Environmental cues may have a critical role in determining the fate of neuroepithelial precursor cells. However, the nature of the extracellular agents that drive precursor cells toward a specific cell fate and the intracellular mechanisms by which these extracellular agents promote such a fate are not well understood.

The ligand-binding subunit of the CNTF receptor α (CNTFRα) is expressed in the embryonic cortical ventricular zone, where the fate of proliferating neuroepithelial precursor cells is determined (*9*). To determine whether activation of the CNTFR in cerebral cortical precursor cells influences their proliferation, differentiation, or survival, we added CNTF to primary cultures of cortical precursors that were derived from embryonic day 14 (E14) or E17 rat embryos and assessed the effects of CNTF on various cellular parameters (*10*). Cortical precursor cells were defined as actively proliferating cells that express the intermediate filament protein nestin, an in vivo marker of neuroepithelial cells (*11*). The cortical precursors were defined further as cells that fail to express markers of differentiated neurons and glia.

Cortical precursors exhibit distinct biological responses to various extracellular stimuli. Precursor cells that maintain cell-cell contact proliferate when exposed to the mitogen basic fibroblast growth factor (bFGF) (*12*). By contrast, cortical precursors differentiate into neurons when they are dissociated into single cells or exposed to neurotrophin-3 (NT-3) or platelet-derived growth factor (PDGF) (*12*–*14*). Unlike bFGF, NT-3, and PDGF, CNTF did not promote proliferation of cortical precursor cells or their differentiation into neurons. Rather, as recently demonstrated for embryonic rat hippocampal stem cells (*14*), CNTF triggered the differentiation of cortical precursors into astrocytes, as indicated by the expression of the astrocyte-specific protein glial fibrillary acidic protein (GFAP) (Fig. 1A). The GFAP-positive cortical cells displayed astrocytic morphological features, including a flat or a stellate appearance (Fig. 1A) (*15*). The proportion of cortical precursor cells that differentiated into astrocytes upon exposure to CNTF increased at E17 compared with E14 (Fig. 1B) (*16*). The increase with age in CNTF responsiveness of the cortical precursor cells parallels the development of astrocytes in vivo, which occurs after neurogenesis. Although the exposure of E14 and E17 cortical cultures to CNTF resulted in a large increase in the number of astrocytes, CNTF had no apparent effect on the survival or proliferation of either precursor cells or astrocytes (Table 1). This suggests that CNTF is not acting selectively on a small proportion of cortical precursors or astrocytes by specifically stimulating their proliferation or survival; rather, it is inducing differentiation of a stable population of cortical precursors along a glial lineage. Expression of GFAP was maintained in the newly generated astrocytes 6 days after removal of CNTF, which suggests that CNTF triggers long-lasting phenotypic changes in cortical precursors (*15*). The cortical precursor cells and the newly generated astrocytes did not express galactocerebroside or the A2B5 surface marker (*15*), which suggests that CNTF is not acting on oligo-

Table 1. Effect of CNTF on E14 and E17 cortical cultures. Results are mean ± SEM (n = 3). DIV, days in vitro; BrdU, bromodeoxyuridine; ND, not determined.

	Cells (% total)					
	E17			E14		
	0 DIV Control	3 DIV		0 DIV Control	4 DIV	
		Control	CNTF		Control	CNTF
Nestin-positive	32.0 ± 5.6	25.0 ± 6.7	25.3 ± 5.8	71.4 ± 5.9	55.0 ± 7.3	58.5 ± 11.5
BrdU*	10.2 ± 1.2	15.0 ± 3.5	16.9 ± 3.2	32.8 ± 3.6	47.0 ± 8.7	43.3 ± 7.4
Neurons	67.5 ± 10.5	59.7 ± 6.5	64.0 ± 7.6	ND	30.8 ± 7.3	33.5 ± 10.7

*A pulse of BrdU was added to the cortical cultures shortly before fixation to assess rate of cell proliferation.

Table 2. Cellular composition of β-galactosidase–expressing clones. Results are mean ± SEM (n = 3).

	Clones (%)			
	Precursor	Neuronal	Astrocytic	Mixed
Control	43.0 ± 5.1	56.3 ± 4.7	0.3 ± 0.3	0.3 ± 0.3
CNTF	21.7 ± 0.3*	18.0 ± 3.2*	52.7 ± 2.2*	7.7 ± 1.9*
PDGF	31.3 ± 4.4	67.7 ± 5.4	1.0 ± 1.0	0

*Treatment of cultures with CNTF, compared with control cultures, reduced significantly the number of precursor and neuronal clones and increased significantly the number of astrocytic and mixed clones (analysis of variance, $P < 0.01$).

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dendrocyte precursor cells to induce the differentiation of type 2 astrocytes. Rather, CNTF appears to promote differentiation of cortical neuroepithelial precursor cells into type 1 astrocytes.

We carried out clonal analysis of E14 cortical cultures to determine whether CNTF promotes astrocytic differentiation of precursor cells that are already committed to the glial lineage or whether CNTF also acts on cells that have the potential to differentiate into both neurons and astrocytes. Proliferating cortical precursors were infected with a replication-defective retrovirus expressing β-galactosidase. The cellular composition of the β-galactosidase–expressing clones was then analyzed by indirect immunofluorescence (Table 2) (*17*). Control cultures contained 43% precursor clones and 56% neuronal clones but essentially no astrocytic clones. Treatment of cultures with CNTF reduced dramatically the percentage of precursor and neuronal clones and led to the appearance of astrocytic clones, which accounted for >52% of the clones. In contrast to CNTF, treatment of cortical cultures with PDGF increased the proportion of neuronal clones to 68% and failed to generate astrocytic clones. The simplest interpretation of these results is that at least 29% of the proliferating E14 cortical precursor cells at the time of plating have the capacity to differentiate into neurons or astrocytes (*17*) and that CNTF drives these precursors to become astrocytes. To rule out the possibility that, by the process of clonal selection, CNTF promotes survival of astrocytes or precursors committed to the glial lineage while killing precursors committed to the neuronal lineage, we carried out clonal analysis of E14 cortical cultures with a retrovirus expressing green fluorescent protein (GFP) to allow the continuous monitoring of individual cells. The survival rate of the clones that were followed in this way until 4 days in vitro (DIV) was >94%, and this rate was not altered by CNTF treatment. Analysis of the cellular composition of the GFP-expressing clones revealed that they behaved similarly to the β-galactosidase–expressing clones. CNTF treatment increased dramatically the number of astrocytic clones and reduced the number of neuronal clones (*17*). These data indicate that clonal selection cannot account for CNTF-induced astrocytic differentiation or for CNTF inhibition of neuronal differentiation. Taken together, these results indicate that CNTF promotes gliogenesis at least in part by instructively driving multipotential precursor cells along the astrocytic glial lineage. The possibility remains that CNTF can also induce cells already committed to the glial lineage to differentiate into astrocytes. In addition, although CNTF inhibits neuronal differentiation of cortical precursor cells, members of the CNTF–leukemia inhibitory factor (CNTF-LIF) family may promote neuronal differentiation of precursor cells in other regions of the developing CNS such as the spinal cord (*18*).

The biological effects of CNTF on responsive cells are mediated by CNTFRα, which, once bound to CNTF, triggers sequential heterodimerization of the two signal-transducing (β) subunits of the CNTFR–LIF receptor β (LIFRβ) and gp130 (130-kD glycoprotein) (*19*, *20*). The differentiation-promoting effects of CNTF on cortical precursor cells were mediated by both β subunits of the CNTFR. Addition of the CNTF-related cytokine interleukin-6 (IL-6) together with the soluble IL-6 receptor (sIL-6R), which act solely through gp130, triggered astrocytic differentiation (Fig. 1B). These results suggest that homodimerization of gp130 in cortical precursor cells mimics the effects of the intact CNTFR. The CNTF-related cytokine LIF, which signals through gp130 and LIFRβ but does not use CNTFRα, also enhanced differentiation of cortical precursors into astrocytes (*15*). CNTF did not promote astrocytic differentiation of cortical precursor cells derived from mice in which the LIFRβ gene was disrupted ($LIFR\beta^{-/-}$) (Fig. 1C). By contrast, the

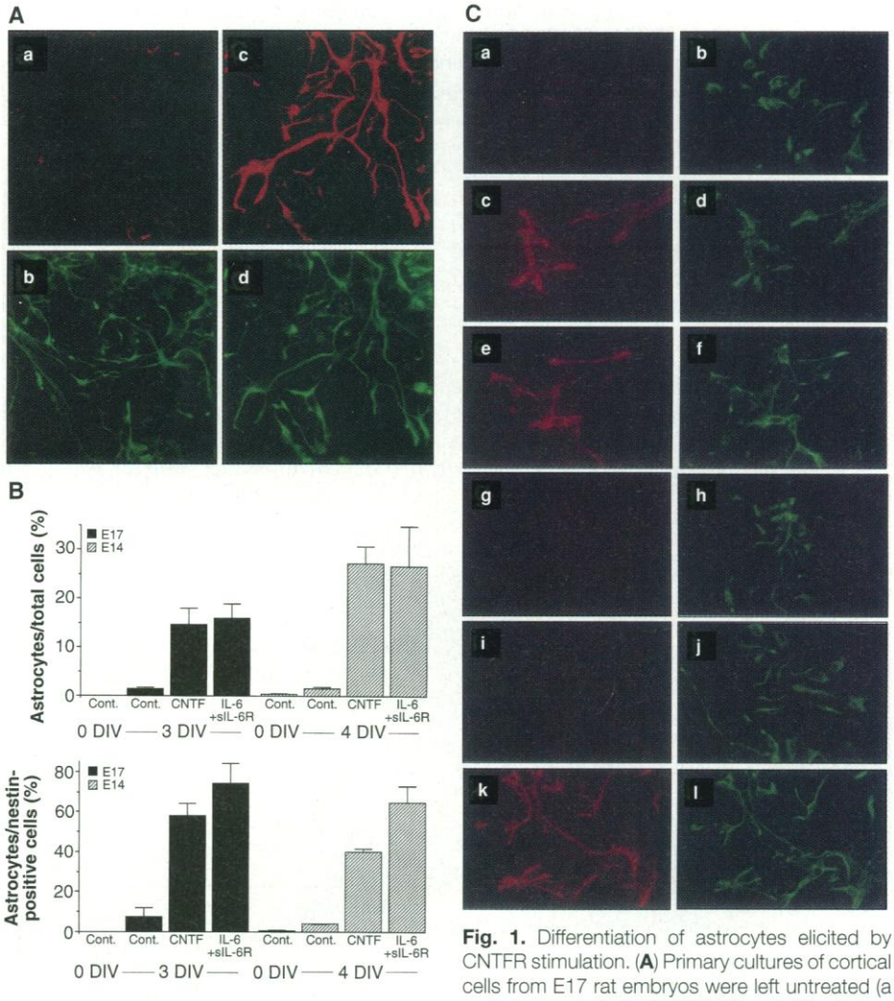


Fig. 1. Differentiation of astrocytes elicited by CNTFR stimulation. (**A**) Primary cultures of cortical cells from E17 rat embryos were left untreated (a and b) or treated with CNTF (100 ng/ml) for 3 days (c and d) and then subjected to indirect immunofluorescence (*38*) with a monoclonal antibody to GFAP [Boehringer Manheim Biochemicals (BMB)] diluted 1:500 (a and c) or a rabbit antiserum to nestin diluted 1:5000 (b and d). (**B**) Quantitative analysis of data from several experiments of the type shown in (A). The number of astrocytes in CNTF-treated or IL-6 + sIL-6R–treated E17 and E14 cultures at 3 and 4 DIV, respectively, were significantly greater than in untreated cultures or than cultures at 0 DIV ($n = 3$; ANOVA, $P < 0.05$). The number of astrocytes is shown as a percentage of the total number of cells (upper) or as a percentage of nestin-positive cells (lower). (**C**) CNTF-induced differentiation of astrocytes is mediated by LIFRβ. Primary cultures of cortical cells from E15 mouse embryos that were wild type (a to f) or homozygous for the mutant LIFRβ allele (g to l) were left untreated (a, b, g, and h) or were treated with CNTF (100 ng/ml) (c, d, i, and j) or IL-6 (20 ng/ml) + sIL-6R (25 ng/ml) (e, f, k, and l) for 3 days. Cultures were fixed and subjected to indirect immunofluorescence with a monoclonal antibody to GFAP (BMB) (a, c, e, g, i, and k) or a rabbit antiserum to nestin (b, d, f, h, j, and l).

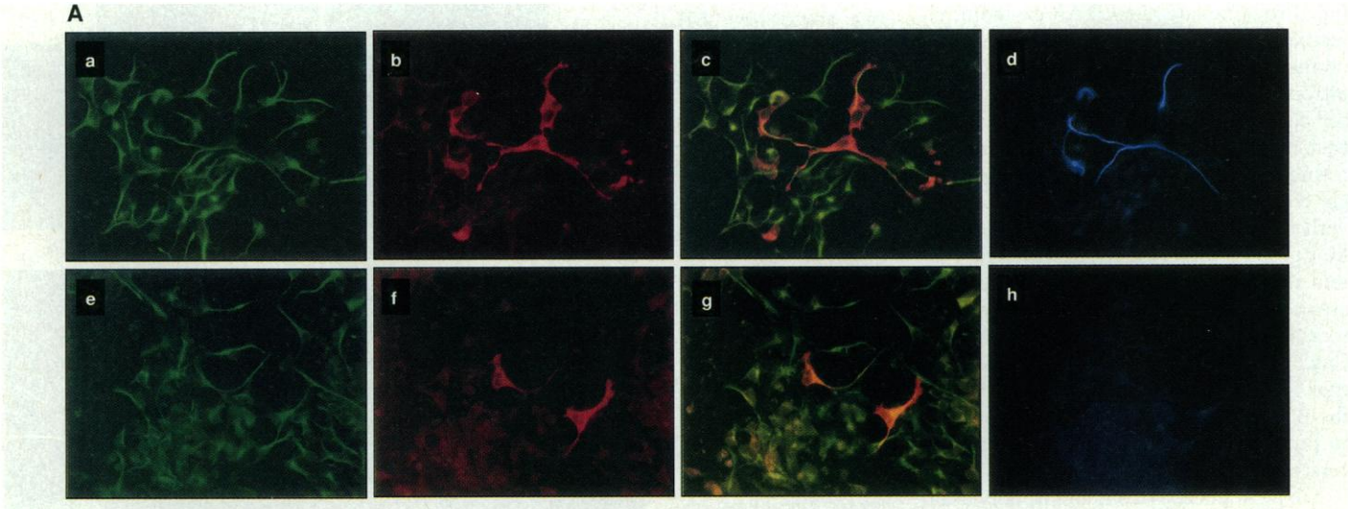
LIFRβ$^{-/-}$ cortical precursor cells were responsive to the addition of IL-6 and sIL-6R (Fig. 1C). These results suggest that gp130 and LIFRβ both contribute to CNTF induction of astrocyte differentiation in vitro.

CNTF and LIF can also induce astrocytic differentiation of precursor cells derived from embryonic mammalian spinal cord (*15*, *18*). The amount of GFAP expression was reduced in LIFRβ$^{-/-}$ mice compared with wild-type or heterozygous mice (*15*). The number of astrocytes in histological sections of the developing spinal cord of LIFRβ$^{-/-}$ mice is also reduced (*21*). Thus, CNTF-related cytokines that act via LIFRβ appear to contribute to the generation of astrocytes in the developing mammalian CNS.

We investigated the mechanism by which CNTFR stimulation initiates the process of astrocyte differentiation. CNTF binding to CNTFRα induces sequential heterodimerization of LIFRβ and gp130, which leads to tyrosine phosphorylation and activation of associated JAK tyrosine kinases (*22–25*). Once activated, the JAKs stimulate phosphorylation of the two β subunits of the CNTFR on specific tyrosine residues, which then serve as docking sites for Src homology 2–containing signaling proteins including the STAT1 and STAT3 transcription factors and the tyrosine phosphatase SHP-2 (*26*). When phosphorylated, four tyrosines within the COOH-terminus of gp130 (Tyr^{767}, Tyr^{814}, Tyr^{905}, and Tyr^{915}) and three tyrosines within the COOH-terminus of LIFRβ (Tyr^{981}, Tyr^{1001}, and Tyr^{1028}) bind to STAT3. Phosphorylated Tyr^{759} within gp130 associates with SHP-2. Once associated with the CNTFR, the STAT proteins and SHP-2 become phosphorylated and activated (*26*, *27*).

We determined the tyrosine residues within the β subunits of the CNTFR that are critical for the CNTF-induced differentiation response. The CNTFR was reconstituted in cortical precursor cells by expressing chimeric proteins that contain cytoplasmic sequences of the β subunits of the CNTFR fused to the extracellular domains of receptor tyrosine kinases. We then determined the effect on astrocyte differentiation of mutations of the specific SHP-2- or STAT-activating tyrosine residues within the cytoplasmic component of these chimeric proteins.

Chimeric proteins containing the cytoplasmic domain of gp130 or LIFRβ fused to the extracellular domain of the epidermal growth factor receptor (EGFR) or the NT-3 receptor TrkC (*28*) were expressed in cortical precursor cells, and the ability of each chimeric protein to trigger astrocyte differentiation in response to EGF or NT-3 was assessed. The ability of these chimeric proteins to mediate ligand-induced activation



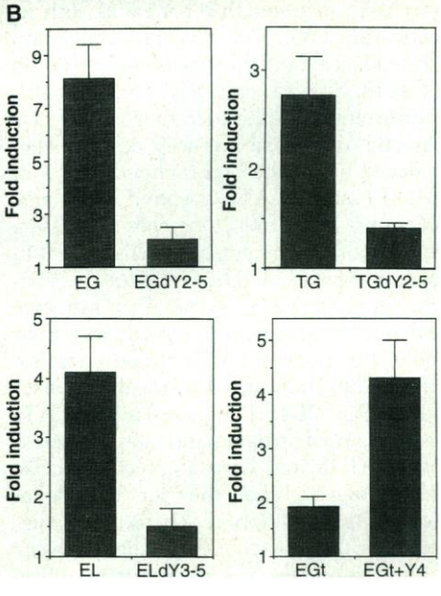


Fig. 2. Requirement of the STAT-activating tyrosine residues within the β subunits of the CNTFR for CNTFR induction of gliogenesis. (**A**) Requirement of the STAT-activating tyrosine residues within the β subunits of the CNTFR for gp130 induction of astrocyte differentiation. Cortical cultures (E14 + 3 DIV) were transfected (*39*) with the EG (a–d) or the EGdY2-5 (e–*h*) plasmid together with the cytomegalovirus (CMV)–β-galactosidase plasmid and then treated with EGF for 24 hours. Cultures were then analyzed by indirect immunofluorescence for the presence of three antigens: β-galactosidase (b, c, f, and g), nestin (a, c, e, and g), and GFAP (d and h) (*40*). The EG and the EGdY2-5 proteins mediated EGF induction of astrocyte differentiation of, respectively, 91.0 and 1.3% of transfected precursor cells. The TG, TGdY2-5, and TGY1-F proteins mediated NT-3 induction of astrocyte differentiation of, respectively, 50, 0, and 55.3% of transfected precursor cells. The EL and ELdY3-5 proteins mediated EGF induction of astrocyte differentiation of 58.5 and 6.7% of transfected precursor cells. The EGt and EGtY4 proteins mediated EGF induction of astrocyte differentiation of 9.5 and 90.4% of transfected precursor cells. (**B**) Requirement of the STAT-activating tyrosines within the β subunits of the CNTFR for CNTF fold induction of the GFAP promoter. Cortical cultures (E17 + 3 DIV) were transfected with the indicated chimeric receptors together with the GFAP luciferase reporter gene and the EF-CAT plasmid to serve as an internal control for transfection efficiency (*41*). The dY2-5 mutation significantly reduced the ability of the EG ($n = 3$; *t* test, $P < 0.05$) and TG ($n = 6$; *t* test, $P < 0.01$) proteins to mediate, respectively, EGF and NT-3 induction of the GFAP promoter. The dY3-5 mutation also reduced the ability of EL ($n = 3$; *t* test, $P < 0.05$) to mediate EGF induction of the GFAP promoter. The Y4 motif increased the ability of the EGt protein to mediate EGF induction of the GFAP promoter ($n = 4$, $P < 0.01$).

of a GFAP-reporter gene was also tested in transient expression assays. The EG (EGFR-gp130), EL (EGFR-LIFRβ), and TG (TrkC-gp130) chimeric receptors mediated astrocytic differentiation of transfected precursor cells (Fig. 2A) and activated the GFAP promoter (Fig. 2B) in response to the appropriate ligand (EGF or NT-3). Thus, when activated, each β subunit of the CNTFR is capable of inducing differentiation of cortical precursors into astrocytes.

Substitution of Phe for Tyr^{759} (Y1-F) within the gp130 component of the TG protein, which selectively impairs its ability to activate SHP-2 in cell lines, did not impede astrocyte differentiation triggered by TG (Fig. 2A). By contrast, the EGdY2-5, TGdY2-5, and ELdY3-5 proteins in which the four STAT-activating tyrosines within gp130 or the three STAT-activating tyrosines within LIFRβ were deleted failed to trigger differentiation of cortical precursor cells into GFAP-positive astrocytes and failed to stimulate the GFAP promoter effectively (Fig. 2, A and B). Another EG chimeric protein (EGt) failed to mediate astrocytic differentiation of cortical precursor cells because of a truncation of gp130 that removed all five COOH-terminal tyrosine residues. However, appending a single STAT-activating gp130 tyrosine motif with the sequence Tyr-Leu-Pro-Gln to the EGt protein conferred to the chimeric protein the ability to mediate EGF-dependent astrocytic differentiation of cortical precursors and to activate the GFAP promoter (Fig. 2, A and B). These findings suggest that the STAT-activating tyrosines within the β subunits of the CNTFR are necessary to promote differentiation of cortical precursor cells into astrocytes.

CNTF induced tyrosine phosphorylation of JAK1, an event that correlates with its activation (Fig. 3A). CNTFR stimulation also induced rapid tyrosine phosphorylation of both STAT1 and STAT3 (Fig. 3B). Consistent with the idea that CNTF acts directly on cortical precursor cells, CNTF-induced tyrosine phosphorylation of STAT1 and STAT3 occurred in a large fraction (30 to 60%) of cells expressing markers of cortical precursor cells but not in neurons (Fig. 3C). The ability of the various chimeric receptors to elicit astrocyte differentiation correlated directly with their ability to trigger STAT tyrosine phosphorylation in transfected cortical precursor cells (Fig. 3D). Phosphorylated STATs were localized predominantly in the nucleus of CNTF-treated cortical precursor cells, which suggests that they are capable of activating transcription. These results suggest that the JAK-STAT signaling pathway has an important role in promoting differentiation of precursor cells into astrocytes.

We tested the importance of the STAT proteins in CNTF-induced differentiation of cortical precursor cells directly by determining the effects of two distinct dominant interfering forms of STAT3 on CNTF-induced astrocyte differentiation. The STAT3F mutant protein can associate with

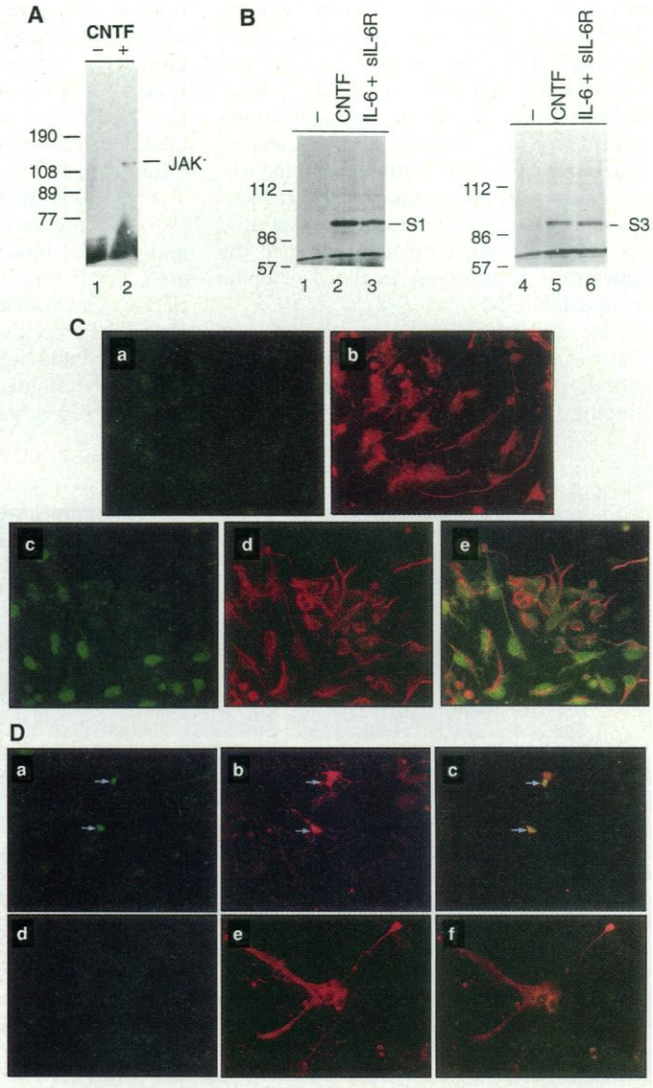


Fig. 3. Requirement for the JAK-STAT signaling pathway for CNTFR induction of GFAP expression in cortical precursor cells. (**A**) Tyrosine phosphorylation of JAK1. Analysis of tyrosine-phosphorylated JAK1 in protein lysates of E17 cortical cultures that were unstimulated (lane 1) or treated with CNTF (100 ng/ml) for 10 min (lane 2) (*42*). Numbers on left are kilodaltons. (**B**) Tyrosine phosphorylation of STAT1 (S1) and STAT3 (S3) in response to CNTFR stimulation. Lysates of E17 cortical cultures that were left untreated (lanes 1 and 4) or were treated with CNTF (100 ng/ml for 15 min; lanes 2 and 5) or IL-6 + sIL-6R (20 and 25 ng/ml for 15 min; lanes 3 and 6) were separated by PAGE, and proteins were immunoblotted with an antiserum to phosphorylated STAT1 (1:10,000; lanes 1 to 3) (*37*) or phosphorylated STAT3 (*43*) (1:7500; lanes 4 to 6). Antibody binding was detected as in (A). (**C**) Cultured cortical precursor cells (E14 + 3 DIV) were left untreated (a and b) or were stimulated with CNTF (100 ng/ml for 15 min; c to e) and subjected to indirect immunofluorescence with antibodies to phosphorylated STAT1 (1:1000; a, c, and e) and a monoclonal antibody to nestin (1:1000; b, d, and e) (*44, 45*). (**D**) The chimeric proteins mediated ligand-specific induction of STAT tyrosine phosphorylation. E17 cortical cultures were transfected with a plasmid encoding the EG (a to c) or the EGdY2-5 (d to f) chimeric protein together with a β-galactosidase expression plasmid. Transfected cultures were treated with EGF (30 ng/ml for 15 min) and then analyzed by indirect immunofluorescence with antibodies to phosphorylated STAT3 (1:1000; a, c, d, and f) and a monoclonal antibody to β-galactosidase (Promega) (1:300; b, c, e, and f). Arrows point to cells that display phosphorylated STAT3 and β-galactosidase immunofluorescence. All cells that contained phosphorylated STAT3 expressed markers of precursor cells. Similar results were obtained with antibodies to phosphorylated STAT1. (**E**) Inhibition of CNTF-induced astrocytic differentiation by dominant interfering forms of STAT3. E14 cortical cultures were transfected with a control vector plasmid (EF-CAT) or an expression plasmid containing STAT3, STAT3F, or STAT3D together with the CMV–β-galactosidase plasmid. Cultures were treated with CNTF (100 ng/ml for 24 hours). Cultures were then fixed and analyzed as in Fig. 2A. STAT3F and STAT3D reduced significantly (n = 3; ANOVA, $P < 0.05$) astrocytic differentiation of transfected precursor cells. For all constructs in the absence of CNTF treatment, <5% of the transfected precursor cells differentiated into astrocytes.

the tyrosine-phosphorylated CNTFR but is not phosphorylated or activated because of the substitution of Tyr^{705} with Phe (*29*). STAT3F is thus thought to interfere with CNTFR activation of endogenous STATs by inhibiting their recruitment to the β subunits of the CNTFR. STAT3D contains a mutation in its DNA-binding domain that inhibits its binding to specific DNA sequences (*29*). Therefore, STAT3D interferes with the action of endogenous STAT1 and STAT3 by forming heterodimers with these proteins, which then fail to bind their cognate DNA-binding sites within CNTF-responsive genes. Both STAT3F and STAT3D blocked significantly the ability of CNTF to induce astrocytic differentiation of cortical precursor cells and to activate the GFAP promoter (Fig. 3E) (*15*, *30*). These results indicate that activation of the JAK-STAT signaling pathway is critical for the ability of the CNTFR to promote differentiation of cortical precursor cells into astrocytes.

We also investigated the mechanism by which tyrosine-phosphorylated STATs induce cortical precursor cells to differentiate into astrocytes. Because CNTF induction of STAT tyrosine phosphorylation occurred with rapid kinetics that preceded CNTF-induced expression of GFAP protein and mRNA (Fig. 4, A and B), and because the GFAP promoter is responsive to CNTF, we considered the possibility that the GFAP gene might contain STAT-binding sites within its regulatory region (*31*). We identified a potential STAT-binding site (TTCCGAGAA) in both the rat and human GFAP promoters (*32*, *33*); 5′ deletion analysis revealed that a 204–base pair region of the GFAP promoter encompassing the potential STAT-binding site has a critical role in mediating CNTFR-induced transcription of the GFAP gene (Fig. 4C). A protein complex that binds this fragment of the GFAP promoter was identified in nuclear extracts of CNTF-treated cortical cultures but not of untreated cultures (Fig. 4D). The complex was supershifted by monoclonal antibodies to STAT1 or STAT3. A mutation in the STAT DNA-binding sequence that disrupted the binding of STAT1 and STAT3 (Fig. 4D) blocked completely the ability of CNTF to activate GFAP promoter-driven reporter gene expression (Fig. 4C). Thus, CNTF induction of GFAP transcription is mediated by STATs binding to a specific site within the GFAP promoter.

In addition to activating the JAK-STAT signaling pathway in cortical precursor

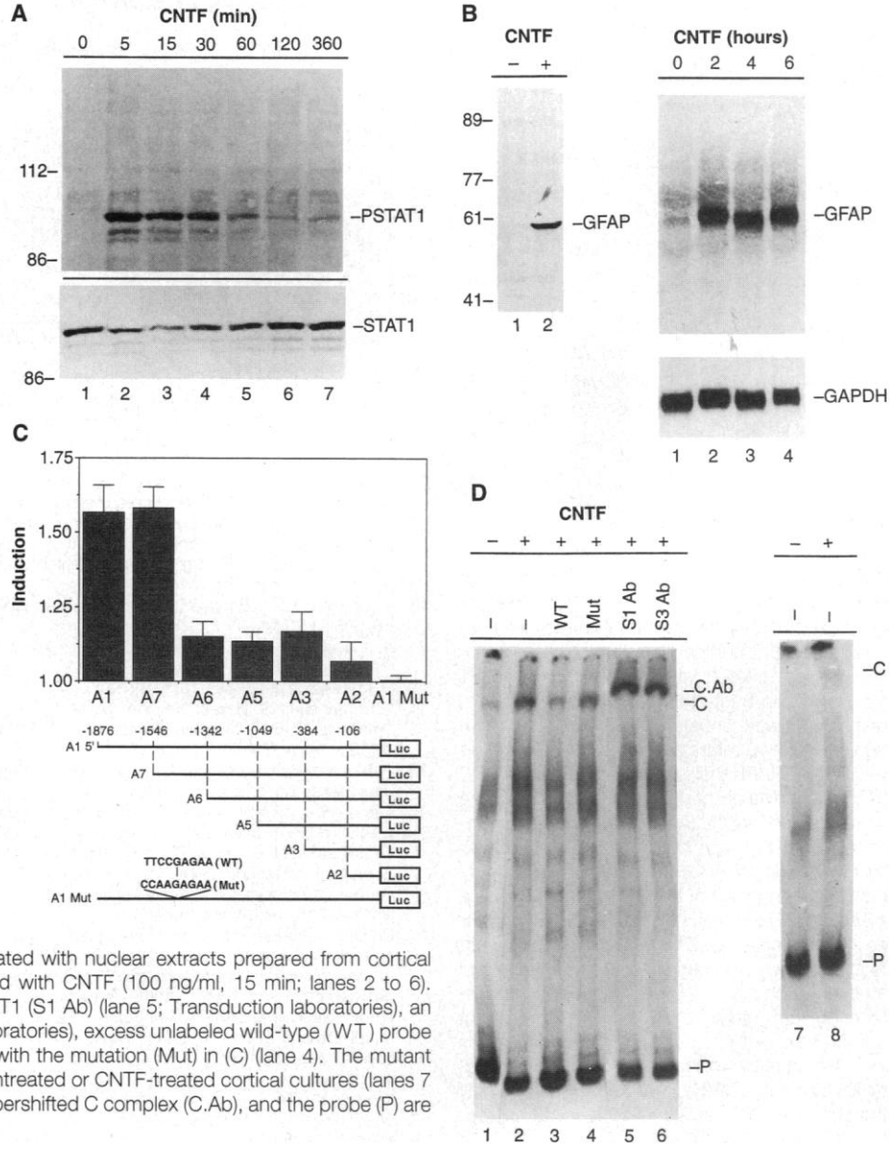


Fig. 4. Activation of transcription of the GFAP gene by STAT1 and STAT3. (**A**) STAT tyrosine phosphorylation occurred with rapid and prolonged kinetics in cells treated with CNTF that preceded expression of GFAP mRNA and protein shown in (B). Lysates of E17 cortical cultures that were untreated or stimulated with CNTF for the indicated times were separated by PAGE and proteins were immunoblotted with the antiserum to phosphorylated STAT1 (1:10,000) or a rabbit antiserum that recognizes STAT1 regardless of its tyrosine phosphorylation status (*27*) (1:5000). Antibody binding was detected by ECL (Amersham) with a secondary antibody conjugated to horseradish peroxidase. (**B**) CNTF induced the expression of GFAP protein and mRNA. Lysates of E17 cortical cultures that were unstimulated (lane 1) or treated with CNTF (100 ng/ml for 3 days; lane 2) were separated by PAGE and proteins were immunoblotted with a monoclonal antibody to GFAP (BMB) diluted 1:1000. Antibody binding was detected by ECL with a secondary antibody conjugated to horseradish peroxidase. RNA was isolated from cortical cultures that were unstimulated or treated with CNTF for the indicated time (right) and analyzed by Northern blot analysis (*46*) with a GFAP cDNA probe or a glyceraldehyde-6-phosphate dehydrogenase (GAPDH) cDNA probe. (**C**) Deletion analysis of activation of the GFAP promoter (shown as fold induction). Cortical cultures were transfected with the wild-type A1 GFAP luciferase reporter gene, with the indicated mutant (deletions A7 to A2) or with the A1Mut plasmid together with the EF-CAT plasmid. CNTF activation of the GFAP promoter was reduced significantly with A6, A5, A3, A2, or the A1mut ($n = 3$; ANOVA, $P < 0.01$) compared with A1. (**D**) Binding of CNTF-activated STAT1 and STAT3 to a specific site within the GFAP promoter (*47*). The −1546 to −1342 fragment of the GFAP promoter was incubated with nuclear extracts prepared from cortical cultures that were left untreated (lanes 1) or treated with CNTF (100 ng/ml, 15 min; lanes 2 to 6). Reaction mixtures also included an antibody to STAT1 (S1 Ab) (lane 5; Transduction laboratories), an antibody to STAT3 (S3 Ab) (lane 6; Transduction laboratories), excess unlabeled wild-type (WT) probe (100 times the labeled probe; lane 3), or the probe with the mutation (Mut) in (C) (lane 4). The mutant probe was also incubated with nuclear extracts of untreated or CNTF-treated cortical cultures (lanes 7 and 8). The specific protein–DNA complex C, the supershifted C complex (C.Ab), and the probe (P) are indicated.

cells, CNTF also stimulated tyrosine phosphorylation of mitogen-activated protein kinase (MAPK) in cortical cultures (Fig. 5A), an event that correlates with its activation. However, inhibition of CNTF induction of MAPK by expression of a dominant interfering form of MAPK kinase (MKK_{KA97}) actually augmented CNTF induction of the GFAP promoter (Fig. 5B). These results suggest that CNTFR activation of the Ras-MAPK signaling pathway opposes the JAK-STAT signaling pathway in promoting gliogenesis and raise the possibility that the Ras-MAPK signaling pathway may be important for proliferation of cortical precursor cells or their differentiation into neurons.

The findings presented in this study indicate that CNTFR stimulation contributes to gliogenesis in the developing mammalian CNS. The importance of GFAP expression for astrocyte differentiation was demonstrated in mice in which the GFAP gene was disrupted. These mice display abnormalities in development of the blood-brain barrier and white matter; they also manifest deficient adaptive responses of the nervous system (*34–36*). In addition to GFAP, CNTFR-activated STATs may stimulate expression of other genes that contribute to the differentiation of astrocytes. Because activation of the STAT proteins does not elicit astrocyte differentiation in proliferating cells outside the nervous system, STAT proteins may cooperate with other transcription factors that are specifically expressed in cortical precursor cells to initiate a program of gene expression that promotes gliogenesis.

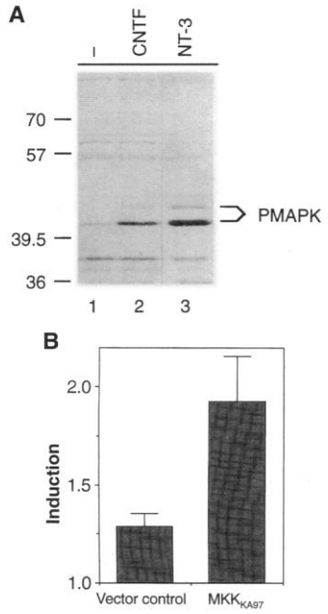


Fig. 5. CNTF-activated MAPK inhibits CNTF induction of GFAP expression. (**A**) CNTF induces tyrosine phosphorylation of MAPK. Lysates from cultures of E17 cortical cells that were unstimulated (lane 1), treated with CNTF (100 ng/ml for 30 min; lane 2), or treated with NT-3 (50 ng/ml for 30 min; lane 3) were separated by PAGE and immunoblotted with a rabbit antiserum that recognizes p42 or p44 MAPK that is phosphorylated at Tyr^{204} (NEB) at a dilution of 1:1000. Antibody binding was detected by ECL (Amersham) with a secondary antibody that was conjugated to horseradish peroxidase. Numbers on left are kD. (**B**) A dominant interfering form of MAPK kinase (MKK) augments CNTF induction (fold) of the GFAP promoter. Cortical cultures (E17 + 3 DIV) were transfected with A1 GFAP luciferase reporter gene and an expression plasmid containing a dominant interfering form of MKK (MKK_{KA97}) or the parent pcDNA3 expression vector. Transfected cultures were left untreated or treated with CNTF (100 ng/ml for 12 hours). MKK_{KA97} increased significantly the ability of CNTF to stimulate reporter expression ($P < 0.05$; $n = 3$).

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10. Cerebral cortices of E14 or E17 Long Evans rats were dissected and enzymatically dissociated in Hanks' buffered salt solution (HBSS; Gibco) with cysteine-activated papain (10 U/ml). Cortices were then washed with trypsin inhibitor, placed in plating medium, and dissociated into single cells with a 5-ml pipette. E14 cortical cells were plated at 2000 cells per square millimeter in L15 medium [International Chemical and Nuclear (ICN)] supplemented with N2 (Gibco) and bFGF (20 ng/ml; Amgen) on cover slips in 24-well plates that had been coated overnight with poly-D-lysine and laminin. E17 cortical cells were plated at 5000 cells per square millimeter in basal Eagle's medium (BME; Sigma) supplemented with 5% fetal calf serum.
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16. The number of astrocytes (see Fig. 1B, lower) was greater in CNTF-treated E17 + 3 DIV cortical cultures than in CNTF-treated E14 + 4 DIV cultures [$n = 3$, analysis of variance (ANOVA), $P < 0.001$]. The number of astrocytes in untreated cultures E17 + 3 DIV and E14 + 4 DIV was similar.
17. Cultures were infected at the time of plating with a replication-defective retrovirus containing a β-galactosidase gene (at a dilution that led to 20 to 50 clones per well in a 24-well plate). Cultures were left untreated or treated with CNTF (100 ng/ml) or PDGF (20 ng/ml) starting at E14 + 1 DIV. Three days later, cultures were fixed and subjected to indirect immunofluorescence with a rabbit antiserum to β-galactosidase (1:500; 5′-3′, Inc.), guinea pig antibody to GFAP (anti-GFAP) and the TuJ1 mouse monoclonal antibody that labels neuron-specific β-tubulin (1:5000). Within each clone, precursor cells were identified as double GFAP-negative and TuJ1-negative cells. Neurons were identified as TuJ1-positive cells, and astrocytes were identified as GFAP-positive cells. The numbers of clones that were analyzed in control, CNTF-treated, and PDGF-treated cultures were 358, 442, and 315, respectively. In clones that were classified as precursor clones, 100% of the cells were precursor cells. In clones that were classified as astrocytic, >80% of the cells were astrocytes and the remainder were precursor cells. In clones that were classified as neuronal, >60% of the cells were neuronal and the remainder were precursor cells. In clones that were classified as mixed, on average 44% of the cells were astrocytes, 17% were neurons, and 39% were precursor cells. The number of cells per clone was similar in control cultures (4.8 ± 0.2), in CNTF-treated cultures (4.6 ± 0.2), and in PDGF-treated cultures (4.5 ± 0.2). Similar results were obtained in cultures that were analyzed with antibodies to β-galactosidase, guinea pig anti-GFAP, and the mouse monoclonal antibody to nestin, where precursor cells were identified as nestin-positive, GFAP-negative cells. Analysis of the various clone cohorts 4 DIV was used as a gauge for the behavior of precursor cells at the time of initial treatment with the various factors. Thus, the minimum percentage of precursor cells that have the potential to differentiate into astrocytes or neurons and that respond to CNTF was calculated as $A - B$, where A is the proportion of precursor cells that have the potential to differentiate into astrocytes, represented by the percentage of astrocyte-containing clones in the CNTF-treated cultures; B is the maximum proportion of precursor cells that may have lost the potential to differentiate into neurons and could differentiate into astrocytes, represented by the percentage of precursor clones in PDGF-treated cultures (where the greatest amount of neuronal differentiation occurred).

    To allow detection of living proliferating precursor cells, cultures were also infected at the time of plating with a retrovirus expressing GFP. GFP expression was detected within the precursor cells at 2 DIV. Cohorts of GFP-expressing clones (with an average of 1.2 cells per clone) were then left untreated (106 clones) or treated with CNTF (104 clones) and were monitored every 3 hours until 4 DIV, when the cultures were fixed and subjected to indirect immunofluorescence. The survival rates of the monitored cell clones at 4 DIV in cultures that were untreated and treated with CNTF were 94.3% and 94.2%, respectively. Within the surviving clones, >98% of cells survived. A subset of GFP-expressing clones (81 in control cultures and 67 in CNTF-treated cultures) was analyzed at 4 DIV for the presence of astrocytes or neurons in separate wells by indirect immunofluorescence with a rabbit antiserum to GFAP or the TuJ1 mouse monoclonal antibody. Control cultures contained no astrocytic clones, whereas 73.5% of clones contained neurons (>80% of cells within these clones were neurons). By contrast, in CNTF-treated cultures 66% of clones contained astrocytes (>80% of cells within these clones were astrocytes) and the number of neuron-containing clones was reduced by 35%.
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28. The EG and TG chimeric proteins contain wild-type cytoplasmic sequences of gp130 [amino acids (aa) 620 to 918] fused to the extracellular domain of the EGFR and TrkC, respectively. The dY2-5 mutation within the EG and TG constructs contains a deletion of gp130 (aa 766 to 918) that removes four COOH-

terminal tyrosine motifs that are critical for gp130 induction of STAT3 in transfected COS cells (*20*). The Y1-F mutation within the TG chimeric protein contains a substitution of Tyr^{759} with Phe. The EL construct contains wild-type LIFRβ cytoplasmic sequences (aa 834 to 1097) fused to the extracellular domain of the EGFR. The dY3-5 mutation contains a deletion of LIFRβ (aa 980 to 1097) that removes three COOH-terminal tyrosine motifs within LIFRβ that are critical for its ability to activate STAT3 in transfected COS cells (*20*). The EGt chimeric protein contains a truncation of gp130 (aa 758 to 918) and EGtY4 is an EGt protein with an appended tyrosine motif (Tyr-Leu-Pro-Gln) derived from Y4 in gp130. The mutations of gp130 and LIFRβ within the chimeric receptors have no effect on the amount of expression of the chimeric receptors (*23*).

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30. Neither mutation of STAT3 (in STAT3F or STAT3D) had an effect on the expression of the protein when tested in transiently transfected COS cells.
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38. Indirect immunofluorescence was done as described (*27*).
39. Transfections were done as described [Z. Xia *et al.*, *J. Neurosci* **16**, 5425 (1995)].
40. In the experiments shown in Fig. 2A, the β-galactosidase signal was detected with a mouse monoclonal antibody to β-galactosidase (Promega) diluted 1:300, followed by a goat antibody to mouse immunoglobulin G (IgG) conjugated to Cy3 [Biological Detection Systems (BDS)]. The nestin signal was detected with a rabbit antiserum to nestin (1:5000) followed by a goat antibody to rabbit IgG conjugated to Cy2 (BDS). The GFAP signal was detected with a guinea pig anti-GFAP (Advanced ImmunoChemical) diluted 1:500, followed by a donkey antibody to guinea pig IgG conjugated to AMCA diluted 1:200 (Jackson ImmunoResearch).
41. Cortical cultures (E17 + 3 DIV) were transfected with a chimeric protein together with the GFAP luciferase reporter gene [containing 1876 nucleotides of the 5′ regulatory region of the GFAP gene fused to the luciferase gene in pGL3 (Promega)] and the EF-CAT plasmid (containing the bacterial chloramphenicol acetyltransferase downstream of the elongation factor 1α promoter) to serve as an internal control for transfection efficiency. Lysates of transfected cultures were left untreated or stimulated with the appropriate ligand [EGF (30 ng/ml) or NT-3 (50 ng/ml)] for 12 hours and were then analyzed for luciferase (Promega kit) and CAT (Dupont kit) activities. Induction with CNTF treatment was determined after luciferase activity was normalized with CAT activity in all transient expression assays in which the GFAP promoter was tested except in the case of transfections with the TG series of chimeric proteins because NT-3 was found to influence expression of the control EF-CAT gene.
42. Immunoprecipitations were done as described (*26*). Protein lysates were immunoprecipitated with an antiserum to JAK1 [Upstate Biotechnology, Inc. (UBI)], separated by SDS–polyacrylamide gel electrophoresis (PAGE), and immunoblotted with antibodies to phosphotyrosine (PY20) (ICN) and 4G10 (UBI). Antibody binding was detected by ECL (Amersham) with a secondary antibody conjugated to horseradish peroxidase. Protein immunoblot analysis was done as described [A. Bonni *et al.*, *Mol. Cell. Neurosci.* **6**, 168 (1995)].
43. The antibodies to phosphorylated STAT3 were generated as described (*37*).
44. The mouse monoclonal antibody to nestin was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, under contract N01-HD-62915 from the National Institute of Child Health and Human Development.
45. Similar results were obtained with the antibody to phosphorylated STAT3 (*15*). CNTF also induced STAT tyrosine phosphorylation in E14 + 0 DIV cortical cultures (*15*).
46. Northern blot RNA analysis was done as described [Y. Sun *et al.*, *Mol. Cell. Neurosci.* **7**, 152 (1996)].
47. DNA mobility-shift assays were done as described (*27*).
48. We thank Regeneron for providing recombinant rat CNTF; T. DeChiara, D. Ezzadine, and C. L. Cepko for the LIFRβ knockout mice; Amgen for bFGF and NT-3; R. MacKay for the rabbit antibodies to nestin; A. Frankfurter for the antibody to TuJ1; R. Vallee for the antibodies to MAP2; K. Nakajima and T. Hirano for the STAT3 plasmids; N. Moghal for the EF-CAT plasmid; E. Krebs for the pCDNA-3-MKK_{KA97} plasmid; D. L. Feinstein for GFAP cDNA; C. A. Walsh for the β-galactosidase–expressing retrovirus; T. D. Palmer and F. H. Gage for the GFP-expressing retrovirus; S. Vasquez for technical assistance; and D. Levy, G. Corfas, J. A. Loeb, T. Vartanian, G. D. Fischbach, B. A. Barres, and members of the Greenberg laboratory for helpful discussions and critical reading of the manuscript. Supported by an NIH RO1 grant (CA43855; M.E.G.) and an MRRC grant (NIHP30-HD 18655). Animal care was in accordance with institutional guidelines.

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Peripheral and Cerebral Asymmetries in the Rat

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Rats learn a novel foraging pattern better with their right-side whiskers than with their left-side whiskers. They also learn better with the left cerebral hemisphere than with the right hemisphere. Rotating an already learned maze relative to the external environment most strongly reduces right-whisker performance; starting an already learned maze at a different location most strongly reduces left-whisker performance. These results suggest that the right-periphery–left-hemisphere system accesses a map-like representation of the foraging problem, whereas the left-periphery–right-hemisphere system accesses a rote path. Thus, as in humans, functional asymmetries in rats can be elicited by both peripheral and cortical manipulation, and each hemisphere makes qualitatively distinct contributions to a complex natural behavior.

Cerebral and peripheral sensory-motor asymmetries in humans have been a central theoretical topic in cognitive neuroscience for more than a century. Today's theories of asymmetries converge on three main ideas. First, asymmetries can involve a general hemisphere dominance for particular skills (*1*) [for example, left hemisphere (LH) specialization for language and right hemisphere (RH) for vision]. Second, asymmetries involve an attentional effect on the contralateral periphery (*2*) (for example, superiority for many language tasks in the right visual field and for many visual tasks in the left visual field). Third, although one hemisphere may be generally dominant for a particular behavioral domain, each hemisphere still contributes a specific kind of processing to it (*3*) (for example, in vision the left hemisphere accesses categorical information, and the right hemisphere accesses metric information). There may be a general computational basis for such functional hemispheric asymmetries. Recent network models have shown that computational systems perform better on complex problems if they segregate them into subproblems that differ in a natural way; this segregation occurs automatically in systems with partially segregated subsystems of different computational configurations (*4*).

The general computational view of the basis for asymmetries suggests that animals other than humans may have behavioral and cerebral asymmetries (*5*). Indeed, some birds and simians have unique mechanisms for specific communicative behaviors in the left hemisphere (*6*). The rat offers a useful case study for research relevant to the general basis for human asymmetries; rats are neither as biologically distant from humans as birds nor as close as simians. Individual rats, in fact, exhibit some neurophysiological and behavioral asymmetries (*7*); however, there is scant evidence that rats as a species have any behavioral asymmetries for natural complex behaviors (*8*).

Our first goal was to establish a popula-

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