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Ciliary Neurotrophic Factor Promotes Müller Glia Differentiation from the Postnatal Retinal Progenitor Pool

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Abstract

Ciliary neurotrophic factor (CNTF) exhibits multiple biological effects during vertebrate retinal development, including regulating the differentiation of photoreceptor cells and promoting the survival and axonal growth of ganglion cells. We report here that in addition to affecting the differentiation of retinal neurons, CNTF also promotes Müller glia genesis in the postnatal mouse retina. In both retinal monolayer and explant cultures, CNTF increases the number of progenitor cells adopting the Müller cell fate. Exogenous CNTF induces phosphorylation of signal transducers and activators of transcription (STAT)3 and extracellular signal-regulated kinase (ERK) among neonatal progenitor cells and newborn Müller cells. In addition, increased levels of endogenous STAT3 and ERK phosphorylation have been observed at around postnatal day 5, coinciding with the peak of Müller glia genesis. Perturbation of STAT and ERK signaling using protein kinase inhibitors and a dominant-negative STAT3 mutant demonstrates that both CNTF-induced STAT and ERK activation are involved in promoting Müller cell production. Moreover, absorbing epidermal growth factor (EGF) signals with a neutralizing antibody did not affect CNTF-induced Müller glial genesis, indicating that the effect of CNTF is not mediated by the known Müller-enhancing activity of EGF. Together, these results support a novel function of CNTF-like cytokines in retinal gliogenesis.

Keywords

Ciliary neurotrophic factor; Retina; Development; Müller glia; Signal transducers and activators of transcription; Extracellular signal-regulated kinase; Epidermal growth factor

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Introduction

The vertebrate retina is composed of six major classes of neuronal cell types and a glial cell type called Müller glia. Cell lineage analyses indicate that the neuronal cells and Müller glia cells are derived from a common pool of progenitors [Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988]. In addition, cell birth date studies have revealed that the development of various retinal cell types follows an evolutionarily conserved chronological order, with Müller glia among the last cell types to become postmitotic and differentiate [Altshuler et al., 1991]. In the postnatal mouse retina, the major cell types being produced by retinal progenitors are rod photoreceptors, bipolar interneurons, and Müller glia, with the peak of Müller cell genesis occurring at postnatal day 4 [Young, 1985]. The somata of mature Müller glia occupy the middle stratum of the retina and extend processes towards both the inner retina to reach the retinal ganglion cell fiber layer and the outer retina to envelop cell bodies of photoreceptors.

Several genes including both transcription factors and cell-cell signaling molecules have been shown to influence Müller glia development, thus supporting the current view that retinogenesis involves both cell-intrinsic determinants and environmental cues [Cepko et al., 1996; Lillien, 1998; Livesey and Cepko, 2001; Hatakeyama and Kageyama, 2004; Yang, 2004]. For example, the basic helix-loop-helix protein NeuroD is involved in suppressing glial fate during retinogenesis since NeuroD-deficient mice show enhanced Müller cell production, whereas misexpression of NeuroD results in the failure of Müller cell fate specification [Morrow et al., 1999]. In contrast, the paired homeobox gene Rax promotes specification of the Müller cell fate since forced expression of Rax, which is normally present in retinal progenitors, results in an increase of Müller cells at the expense of retinal neurons [Mathers et al., 1997; Furukawa et al., 1997, 2000]. Signals mediated by the transmembrane receptor Notch also play a critical role in Müller glia development, as misexpression of a constitutively active Notch mutant promotes the Müller fate [Bao and Cepko, 1997; Furukawa et al., 2000]. Furthermore, misexpression of the two Notch gene effectors, Hes1 and Hes5, as well as the Hes-related gene Hesr2, enhances Müller glia production in the postnatal retina [Hojo et al., 2000; Furukawa et al., 2000; Satow et al., 2001]. These studies are consistent with experiments showing that Rax protein upregulates transcription of Notch and Hes1 in retinal cells [Furukawa et al., 2000]. The transmembrane epidermal growth factor receptor (EGFR)-mediated signals also influence Müller glia genesis since overexpression of EGFR in postnatal retinal progenitors augments Müller glia genesis in vivo [Lillien, 1995].

The cytokine ciliary neurotrophic factor (CNTF) has been shown to affect neuronal differentiation and survival through a tripartite receptor complex [Ip, 1998]. Ligand binding activates receptor-associated cytoplasmic Jak kinases, which in turn phosphorylate latent 'signal transducers and activators of transcription' (STAT) proteins to result in STAT dimer formation and nuclear translocation [Darnell, 1997]. In addition to the Jak-STAT signaling pathway, CNTF also triggers phosphorylation of the extracellular signal-regulated kinase (ERK, also known as the p42/44 MAP kinase) in certain cell types [Bonni et al., 1997]. In cortical neuronal cultures, CNTF promotes differentiation of astrocytes [Bonni et al., 1997; Rajan and McKay, 1998]. However, activation of the STAT factors rather than ERK appears

to mediate the CNTF-dependent gliogenesis of astrocytes [Ware et al., 1995; Bonni et al., 1997; Koblar et al., 1998; Nakashima et al., 1999].

In the developing retina, CNTF displays potent effects on neuronal differentiation and survival. In both rat and mouse retinas, CNTF as well as leukemia inhibitory factor (LIF), another CNTF-like cytokine, strongly inhibit rod photoreceptor cell differentiation [Kirsch et al., 1996; Ezzeddine et al., 1997; Neophytou et al., 1997; Schulz-Key et al., 2002]; whereas in the chicken retina, CNTF promotes development of a subset of cone photoreceptors [Fuhrmann et al., 1995; Kirsch et al., 1996; Xie and Adler, 2000]. Furthermore, CNTF enhances the expression of bipolar neuron markers in the rat retinal culture [Ezzeddine et al., 1997; Schulz-Key et al., 2002; Bhattacharya et al., 2004]. Here, we report that in addition to its effects on photoreceptor and bipolar cell differentiation, CNTF also promotes Müller glia genesis from the postnatal retinal progenitor pool. We provide evidence that the effect of CNTF on Müller glia production is dependent on activation of both the Jak-STAT and ERK pathways.

Materials and Methods

Animals, Growth Factors, Signaling Inhibitors, and Antibodies

All retinal tissues were derived from CD1 mice obtained from Charles River (Wilmington, Mass., USA). Recombinant rat CNTF and mouse epidermal growth factor (EGF) were purchased from PeproTech Inc. (Rocky Hill, N.J., USA). Recombinant human LIF was obtained from R&D Systems (Minneapolis, Minn., USA). The specific inhibitor U0126 of MEK1 and MEK2 was purchased from Cell Signaling Inc. (Beverly, Mass., USA). The specific inhibitor of Jak2 (AG490) was purchased from Calbiochem (San Diego, Calif., USA). The antibody sources and dilution ratios for different assays are summarized in table 1.

Retinal Cultures

For monolayer cell cultures, retinas were dissected from postnatal 0 (P0) or P3 CD1 mice and dissociated with trypsin as previously described [Altshuler and Cepko, 1992]. Retinal cells were plated at 2,500 cells/mm² on 8-well LabTech slides coated with poly-D-lysine (2 µg/cm²) and laminin (1 µg/cm²). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 (1:1) with 10 mM HEPES pH 7.0 and N2 supplement (Gibco Life Technologies, Rockville, Md., USA) containing 1% fetal calf serum in the presence or absence of 10 ng/ml CNTF. 5-Bromo-2'-deoxyuridine (BrdU) was added to the medium at 20 µM for the last 12 h of the culture period before harvesting.

For explant cultures, dissected retinal cups were placed on poly-carbonate filter discs (Millipore, Bedford, Mass., USA) as previously described [Ezzeddine et al., 1997; Zhang and Yang, 2001] and cultured in DMEM/F12 (1:1) with 10 mM HEPES pH 7.0 and N2 supplement containing either 1% FCS (fig. 1B, 4 A, 5 D) or no serum. After a specified number of days in vitro (DIV), explants were detached from the filters and dissociated with trypsin [Altshuler and Cepko, 1992]. Dissociated cells were plated on glass slides coated with poly-D-lysine at 37°C for 1–2 h before fixation and immunostaining. For pulse-labeling

of explants (fig. 4A), BrdU was added to the culture media to a final concentration of 20 μ M at the beginning of the culture for 12 h, then explants were washed extensively and transferred to fresh filters and media for 5.5 days with or without CNTF. For experiments using the MEK1/2 or Jak2 inhibitors, U0126 or AG490 was added to the medium to reach a final concentration of 10 μ M at the beginning of the culture period, respectively. For studies using anti-EGF, the neutralizing antibody was added to the medium to 10 μ g/ml at the beginning of the culture and once again after 3 days.

Electroporation of Retinal Explants

Dissected retinal cups without pigmented epithelium and lens were submerged in 1 μ g/ μ l DNA in PBS and subjected to 5 electric pulses at 8 V with 10 ms duration and 950 ms intervals using a BTX ECM830 square wave generator (San Diego, Calif., USA) and paddle electrodes. The STAT3EE [Horvath et al., 1995] mutant cDNA was cloned into the LIA, murine retroviral vector downstream of the long terminal repeat promoter [Bao and Cepko, 1997] and the parental vector LIA was used as a control. Both LIA-STAT3EE and the control LIA vector were co-electroporated with a DNA construct that expresses enhanced green fluorescent protein (GFP) from the chicken β -actin promoter [Matsuda and Cepko, 2004]. Transfected explants were further cultured in vitro as described above.

Western Blot Analysis

Protein extracts were collected in lysis buffer (50 mM Tris-HCl pH 7.8, 250 mM NaCl, 0.5 % NP40) containing 1 mM of orthovanadate and protease inhibitors from dissociated retinal cell cultures. Homogenates were centrifuged for 5 min at 5,000 *g*, then 20 μ g of the supernatant were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membrane (Millipore) by electroblotting. The membranes were blocked for 1 h at room temperature or overnight at 4°C in TBS (20 mM Tris-HCl pH 7.6, 150 mM NaCl) containing 5% skim milk, followed by incubation for 1 h at room temperature with specific primary antibodies (table 1) in blocking solution. After incubation, the membranes were washed 3 times with TBS containing 0.1% Tween-20, and then incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated to horseradish peroxidase (Amersham; table 1). After successive washing with TBS containing 0.1% Tween-20, the peroxidase activity was detected using the Enhanced Chemiluminescence System (Amersham).

Immunostaining and Data Analyses

To assay STAT3 and ERK phosphorylation by immunostaining and confocal imaging, whole retinal cups from P3, P4, and P5 were placed on culture plate inserts (Millipore) with the ganglion cell layer up and cultured in 1.5 ml of DMEM/F12 (1:1) containing 10 mM HEPES pH 7.0 and N2 supplement. After 3 h of incubation at 37°C, explants were treated with or without 100 ng/ml CNTF for 15 min before overnight fixation with 4% paraformaldehyde, cryoembedding in OCT compound and sectioning. Monolayer retinal cultures or dissociated cells from retinal explants were fixed with 4% paraformaldehyde in PBS for 15–20 min before immunostaining.

Immunofluorescent staining was carried out as previously described [Zhang and Yang, 2001; Rhee and Yang, 2003]. For double staining with anti-phospho-STAT3 and anti-phospho-

ERK, cryosections were washed 3 times with PBS and then incubated in deionized 95% formamide/1.5% 20 × SSC pH 7.0 for 10 min at 70°C. Following formamide treatment, sections were washed twice with PBS containing 0.1% Tween 20 for 10 min at 4°C before binding with the primary antibodies. Fluorescent staining signals of monolayer cells were captured with a Nikon E800 microscope equipped with a SPOT II digital camera. Confocal imaging of retinal sections was performed using Leica TCS-SP or Zeiss Renaissance 410 confocal laser scanning microscopes as 1- or 2-μm optical sections.

ImagePro Plus software (Media Cybernetics) was used to measure and analyze fluorescent staining signals in monolayer cells. Quantification of marker staining is expressed as the mean ± SEM after statistical analyses using the Mann-Whitney U test or Student's t test. Probability values <0.05 were considered significant. A minimum of 3 independent experiments (n = 3) with a total of 6 separate fields (640 ± 18 cells/field) of each condition was quantified. The same cut-off thresholds for fluorescent staining signals were used for a given antibody for all samples in the same experiment.

Results

Effects of CNTF on Müller Glia Production

Previous reports have demonstrated that CNTF induces astrocyte differentiation in primary cortical neuronal cultures and in the optic nerve [Bonni et al., 1997; Rajan and McKay, 1998; Nakashima et al., 1999; Mi and Barres, 1999]. However, it is yet to be determined if CNTF promotes the glial cell fate of retinal progenitor cells. We thus examined the effect of CNTF on the development of Müller glia cells in early postnatal retinal cultures. Retinal cells derived from P0 and P3 mice were cultured as monolayers in the presence or absence of exogenous CNTF (10 ng/ml) (fig. 1A). In both P0 and P3 cultures grown in the presence of CNTF (10 ng/ml), the proportions of the glial fibrillary acidic protein (GFAP)-positive cells among the total cell population were significantly increased (fig. 1A). CNTF-dependent increases in GFAP marker staining were readily detectable after 1.5 DIV and by 3 DIV, CNTF treatment resulted in a nearly 10-fold increase in the percentage of GFAP⁺ cells compared to untreated controls (fig. 1A).

Since GFAP is expressed by astrocytes and activated Müller cells [Bonni et al., 1997; Wang et al., 2002], it is plausible that CNTF merely enhanced transcription of the GFAP gene in monolayer cultures rather than promoted the Müller cell fate. To rule out this possibility, we quantified both GFAP and an additional Müller cell marker, the cellular retinal binding protein (CRALBP) [De Leeuw et al., 1990] in retinal explants in vitro. Consistent with the results obtained from monolayer cultures (fig. 1A), the percentage of GFAP⁺ cells in P0 retinal explants cultured for 6 DIV in the presence of 10 ng/ml CNTF was significantly increased from 0.7 to 8.2% (fig. 1B). Similarly, the percentage of CRALBP⁺ cells rose from 1.5 to 6.7% in CNTF-treated explants (fig. 1B). We also found that the CNTF family cytokine LIF similarly enhanced GFAP⁺ cells in postnatal retinal explants cultured from P0 to P6, whereas EGF had no effect on GFAP⁺ cells [data not shown].

Thus, these results demonstrate that CNTF promotes Müller glial cell production in vitro in both dissociated cell cultures and explants of neonatal mouse retina.

Activation of STAT3 and ERK in Progenitor and Müller Cells

In the mature rodent retina, CNTF-like cytokines stimulate STAT and ERK phosphorylation in retinal ganglion cells and Müller cells [Peterson et al., 2000; Wahlin et al., 2000]. In the postnatal developing retina, distribution of cytokine signaling components suggest that both progenitors as well as postmitotic neurons and glia may respond to cytokine signals [Rhee and Yang, 2003; Zhang et al., 2003]. To examine if the Jak-STAT and ERK signaling pathways are activated by CNTF in the neonatal mouse retina during development, we treated freshly dissected retinal explants with CNTF and performed immunostaining using antibodies that recognize phosphorylated STAT3 and ERK proteins.

Confocal image analyses showed that at P3, there were detectable yet relatively low levels of phospho-STAT3 in the differentiated retinal ganglion cell layer and in the accumulating postmitotic amacrine cell population (fig. 2A). No endogenous phospho-ERK staining signals were detected (fig. 2A). Upon exposure to exogenous CNTF (100 ng/ml) for 15 min, the phospho-STAT3 staining signals intensified in the ganglion cell layer and in the outer retina where postmitotic photoreceptor cells resided (fig. 2B). Furthermore, a population of cells in the middle of the retina, where retinal progenitors and nascent Müller cells were located, stained positive for phospho-STAT3 (fig. 2B). CNTF stimulation also resulted in intense phospho-ERK staining in the ganglion cell layer, the outer retina, and the ventricular zone of the retina (fig. 2B). Merging of phospho-STAT3 and phospho-ERK staining images revealed costaining of cells in all three zones of the P3 retina. At P4, double staining using antibodies against the progenitor marker proliferating cell nuclear antigen (PCNA) and phospho-ERK showed that many PCNA⁺ progenitors occupying the central retina (fig. 2C) contained phospho-ERK after CNTF treatment (fig. 2D).

By P5, higher levels of endogenous phosphorylation of STAT3 were observed (fig. 2E) compared to younger retinas (fig. 2A, C). Stimulation with CNTF further expanded the cell population containing phospho-STAT3, especially in the inner nuclear layer (fig. 2F). Likewise, high levels of ERK phosphorylation were observed in P5 retinas in the absence of exogenous CNTF treatment (fig. 2G). These phospho-ERK⁺ cells were mostly localized in the inner nuclear layer and colabeled with the Müller cell marker glutamine synthetase (fig. 2G). CNTF stimulation of P5 retina however did not significantly change the phospho-ERK staining pattern, except for slightly more intense staining in the outer nuclear layer (fig. 2H).

Together, these results show that CNTF triggers phosphorylation of both STAT3 and ERK in early postnatal retinal cells. Furthermore, there is a transition from relatively low levels of endogenous STAT3 and ERK phosphorylation prior to P3 to higher levels of these phospho-proteins during the peak period of Müller glia genesis.

Enhanced Müller Genesis by CNTF from the Progenitor Pool

We next investigated if CNTF had increased the number of precursor cells acquiring GFAP immunoreactivity by stimulating proliferation of early postnatal progenitor cells. Dissociated retinal cultures were treated with CNTF for various lengths of time and then labeled with DNA synthesis precursor BrdU for 12 h before harvesting. Immunocytochemical detection of incorporated BrdU showed no significant difference in the percentage of BrdU-labeled

cells in the presence or absence of CNTF (fig. 3A). Similar BrdU incorporation assays were performed in P0 retinal explants cultured for different lengths of time and labeled for 24 h before harvesting (fig. 3B). CNTF did not affect BrdU incorporation in retinal explants between P0–P1 and P2–P3 (fig. 3B). However, between P5 and P6, CNTF-treated explants showed a small yet statistically significant increase of BrdU incorporation compared to the controls (from $0.94 \pm 0.16\%$ to $1.85 \pm 0.18\%$) (fig. 3B). These data indicate that CNTF does not assert a major influence on progenitor cell proliferation in the early postnatal retina. We also examined if CNTF acts as a mitogen for differentiated Müller cells. In enriched Müller cell cultures [Hicks and Courtois, 1990], CNTF treatment did not affect BrdU incorporation [data not shown], thus ruling out the possibility that CNTF specifically stimulates Müller cell proliferation.

We next examined if CNTF promoted retinal progenitors to adopt the Müller glial cell fate. P0 progenitor cells were thus pulse-labeled with BrdU and then monitored for their subsequent differentiation at P6. In control explants not treated with CNTF, approximately 1.7% of the BrdU-labeled progenitor cells became GFAP⁺ by 6 DIV. In contrast, nearly 13% of the BrdU-labeled cells differentiated as GFAP⁺ cells in CNTF-treated explants (fig. 4A). Similarly, the percentage of BrdU⁺ cells colabeled with CRALBP increased to 20% in the presence of CNTF compared to 3% found in controls (fig. 4A). Furthermore, in retinal explants cultured for 6 DIV, cells labeled with BrdU during the last 24 h of culture also showed 11-fold increase of GFAP⁺ cells under the influence of CNTF compared to nontreated controls (2.9 ± 1.6 to $36.7 \pm 3.2\%$) (fig. 4B). These results thus indicate that CNTF signals have biased postnatal retinal progenitor cells to differentiate into Müller cells in vitro.

Effects of Perturbing MEK-ERK and Jak-STAT Pathways on Müller Glia Genesis

We next used pathway-specific inhibitors to examine whether activation of STAT and/or ERK mediated the effects of CNTF on Müller glia development. ERK signaling was blocked with U0126, a specific inhibitor of the protein kinases MEK1 and MEK2, which act upstream of ERK [Favata et al., 1998]. U0126 is a potent inhibitor of ERK signaling, as evidenced by the fact that in dissociated retinal cultures, 10 μ M U0126 completely blocked ERK phosphorylation induced by acute exposure to CNTF or EGF [data not shown]. The inclusion of 10 μ M U0126 in P0 retinal explants cultured for 6 DIV did not alter the percentage of GFAP⁺ Müller glia among total cells in the absence of exogenous CNTF (fig. 5A). In contrast, U0126 significantly reduced the number of GFAP⁺ cells from 3.1 to 0.9% in explants treated with 100 ng/ml CNTF (fig. 5A). Similarly, U0126 also blocked CNTF-induced increase of the Müller cell marker cyclin D3 [Hojo et al., 2000] in retinal explants (fig. 5B).

The Jak-STAT signaling pathway was disrupted using the Jak2 kinase inhibitor AG490 [Meydan et al., 1996; Neilsen et al., 1997; Wang et al., 1999]. Western blots of P0 retinal monolayer cells showed that at 10 μ M AG490 inhibited CNTF-induced STAT3 phosphorylation but had no effect on CNTF-induced ERK phosphorylation (fig. 5C). In the presence of AG490 inhibitor, both CNTF-dependent increases of GFAP⁺ and CRALBP⁺ cells were significantly reduced to near endogenous levels (fig. 5D).

To further confirm the role of the Jak-STAT pathway in retinal gliogenesis, we used a dominant-negative STAT3 mutant (STAT3EE) that contains two amino acid residue substitutions in the DNA-binding domain and prevents STAT dimers from activating transcription [Horvath et al., 1995]. A STAT3EE-expressing DNA construct was co-electroporated with a GFP-expressing plasmid into P0 retinal explants. After 6 DIV, the STAT3EE-transfected cells, which were labeled with GFP, showed 50% decrease of the Müller cell marker cyclin D3 in the absence of exogenous ligands compared to controls (21.0 ± 1.7 to 10.4 ± 2.0%) (fig. 5E).

Together, the results of pathway-specific inhibitors support that both the Jak-STAT and MEK-ERK pathways participate in CNTF-induced gliogenesis in the retina, whereas the result of STAT3 mutant perturbation indicates that STAT3 activity is required for Müller glia genesis during normal development.

CNTF-Dependent Müller Production Is Not Mediated by EGF

Elevated EGFR expression among postnatal retinal progenitor cells has been shown to increase Müller cell production [Lillien, 1995]. To examine if the effects of CNTF on Müller glia genesis are mediated through EGF, we used an EGF-neutralizing antibody [Li et al., 1999], which completely abolishes robust ERK phosphorylation induced by 10 ng/ml EGF in monolayer retinal cultures [data not shown]. Inclusion of this anti-EGF antibody at 10 µg/ml in retinal explant cultures did not alter the percentage of GFAP⁺ (fig. 6A) or cyclin D3⁺ (fig. 6B) cells in the presence or absence of exogenous CNTF. Therefore, the Müller promotion effects of CNTF are not likely to be mediated by EGF signals present in the retina.

Discussion

Our studies demonstrate that CNTF promotes the Müller glial cell fate in the developing mouse retina. This effect of CNTF on Müller glia production is consistent with the astrocytic promoting activity of CNTF found in other regions of the central nervous system [Bonni et al., 1997; Rajan and McKay, 1998; Nakashima et al., 1999; Mi and Barres, 1999; Mi et al., 2001]. In both cortical and retinal cultures, it has been shown that cytokine-activated STATs can directly regulate transcription of the GFAP gene, which is expressed by astrocytes and by activated Müller glia [Sun et al., 2001; Wang et al., 2002]. However, since CNTF treatment of retinal cells not only enhanced expression of GFAP, but also enhanced expression of two other Müller cell markers, CRALBP and cyclin D3, we conclude that CNTF indeed promotes the Müller cell fate rather than simply upregulate GFAP expression.

We have demonstrated that CNTF has no mitogenic effects in retinal monolayer cells and explants between P0 and P5. Between P5 and P6, however, 100 ng/ml CNTF caused a slight yet statistically significant enhancement (0.9%) of cell proliferation in explants cultured in the absence of serum. We cannot rule out the possibility that this minor mitogenic effect of CNTF may contribute to enhanced Müller production. However, because CNTF significantly promotes P0 progenitor cells towards Müller cell fate without affecting early cell proliferation and CNTF does not induce mature Müller glial cells to reenter the cell cycle, the late increase of cell proliferation cannot account for the entire Müller promotion effect

by CNTF. We thus conclude that CNTF affects Müller cell specification and/or differentiation from the postnatal retinal progenitor pool.

In the mature retina, CNTF-like ligands trigger STAT and ERK phosphorylation in ganglion cells and Müller glia [Peterson et al., 2000; Wahlin et al., 2000], and assert potent neural protective effects on photoreceptors and ganglion cells [LaVail et al., 1992, 1998; Meyer-Franke et al., 1995; Cayouette et al., 1998; Cui et al., 1999; Jo et al., 1999; Chong et al., 1999; Weise et al., 2000; Bok et al., 2002]. Recent studies have shown that in the developing retina, progenitor cells as well as postmitotic retinal neurons and glia are likely to be responsive to cytokine-signals since they express cytokine signaling components [Rhee and Yang, 2003; Zhang et al., 2003]. We demonstrate here that CNTF induces STAT3 and ERK phosphorylation in retinal progenitor cells and nascent Müller cells during the peak gliogenic period. In contrast to cortical neurons, where the activation of the Jak-STAT but not MEK-ERK pathway is required for the astrocytic promoting activity of CNTF [Bonni et al., 1997], our results demonstrate that inhibiting either ERK or STAT3 activation in the retina hinders the ability of CNTF to promote Müller glia production. Moreover, the effect of the dominant negative STAT3 mutant in the absence of exogenous ligand indicates that endogenous STAT3 activity is required for Müller glia genesis. Therefore, both STAT and ERK activation contribute to progenitor cell fate specification toward Müller glia and/or their subsequent differentiation.

Several lines of evidence support that members of the CNTF family of cytokines play critical roles during retinal neurogenesis. Neutralizing antibodies against CNTF or LIF revealed that endogenous CNTF and LIF inhibit rod development in the postnatal retina, even though these two proteins could not be detected in the retina by immunocytochemistry [Neophytou et al., 1997]. In addition, retinal explants derived from CNTF α - or LIFR β -deficient mice develop excessive numbers of rod photoreceptors in vitro [Ezzeddine et al., 1997]. Moreover, it has been shown that embryonic retinas produce a molecule(s) that signals through the gp130/LIFR β complex to inhibit postnatal progenitor cells from becoming rod photoreceptor cells [Belliveau et al., 2000]. In this study, we demonstrate by immunostaining that there is a marked increase of endogenous phospho-STAT3 and phospho-ERK between P3 and P5. Similar elevation of phospho-STAT3 has also been detected at P7 and P10 by Western blot analyses in the absence of exogenous cytokines [Rhee et al., 2004]. These data suggest that cytokine signaling is dynamic during retinogenesis and that there may be an increase of endogenous cytokine signals in vivo coinciding with the peak of Müller cell birth and differentiation. Consistent with our observations, it has been reported that newborn Müller glia begin to express LIF in vivo [Neophytou et al., 1997] and thus may be one source of endogenous cytokines in the postnatal retina. This increased expression of endogenous cytokine derived from nascent Müller glia may play a role in promoting uncommitted progenitor cells to adopt the glial fate as well as in facilitating terminal differentiation of Müller cells to establish the glial scaffold necessary for retinal maintenance and function.

Several studies suggest that factors that prolong or sustain progenitor cell proliferation during retinogenesis impact the production of Müller glia. For example, several Müller promoting genes such as Rax, Hes1, and Hes5 are required for sustaining retinal progenitor

cell proliferation [Ishibashi et al., 1995; Tomita et al., 1996; Matthers et al., 1997]. Furthermore, the EGFR, which enhances Müller glia genesis when overexpressed [Lillien, 1995], normally mediates mitogenic signals in the developing retina [Lillien and Cepko, 1992]. In fact, recent investigations suggest that differentiated Müller glia retains certain progenitor-like properties such as proliferative and neurogenic potentials upon injury or stimulation with growth factors [Dyer and Cepko, 2000; Fischer and Reh, 2001]. In this study, we demonstrate that CNTF treatment induces ERK phosphorylation without dramatically affecting cell proliferation in the retina, and CNTF-induced ERK activation is required for Müller cell differentiation. Furthermore, we show that the Müller-promoting activity of CNTF is not mediated by EGF, and EGF by itself does not promote Müller fate despite its potent effect on ERK phosphorylation [data not shown]. Therefore, ERK activation induced by cytokine signals appears to play a different role in cell fate specification and proliferation than ERK phosphorylation caused by a typical mitogen such as EGF. Recently, it has been reported that EGF signaling controls the competence of cortical progenitors to interpret LIF signals and to adopt the astrocyte fate [Viti et al., 2003]. Whether a similar mechanism is involved in retinal gliogenesis remains to be investigated.

In summary, we provide evidence that CNTF enhances Müller glial genesis during postnatal mouse retinal development, and that both cytokine-induced STAT and ERK activation are involved in Müller cell differentiation. In contrast, recent evidence indicates that CNTF-mediated rod photoreceptor suppression involves STAT but not ERK activities [Ozawa et al., 2004; Rhee et al., 2004; Zhang et al., 2004]. Our study thus reveals a novel function of CNTF-like cytokines in retinal gliogenesis in addition to their known effects on neuronal differentiation.

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Fig. 1.

Effects of CNTF on Müller glia genesis in the postnatal retina. Quantifications of Müller cell markers in vitro are shown. **A** Percentages of GFAP⁺ cells among total cells in dissociated P0 and P3 retinal monolayer cultures at various times in the presence (black bars) or absence (white bars) of CNTF (10 ng/ml). **B** Percentages of CRALBP⁺ (gray) and GFAP⁺ (white) cells among total cells in P0 retinal explants cultured for 6 DIV in the presence or absence of CNTF (10 ng/ml). Results are presented as the mean $\pm$ SEM of 3 independent experiments ($n = 3$). The asterisks * and ** indicate p values <0.05 and <0.01 when compared to nontreated samples, respectively.

Fig. 2.

Distribution of phosphorylated STAT3 and ERK in the postnatal retina. Photomicrographs show confocal immunostaining images of P3 (**A, B**), P4 (**C, D**), and P5 (**E–H**) tissue sections derived from retinal explants not treated (**A, C, E, G**) or treated (**B, D, F, H**) with CNTF (100 ng/ml) for 15 min. **A, B** Merged staining images of phospho-STAT3 (red) and phospho-ERK (green); **C, D** merged staining images of progenitor cell marker PCNA (green) and phospho-ERK (red). **E, F** Confocal images of phospho-STAT3 (red). **G, H** Merged staining images of Müller marker glutamine synthetase (GS, green) and phospho-ERK (red). White arrows indicate double-stained cells. Scale bar in (**E**) represents 50 μ m for all panels. *gcl* = Ganglion cell layer; *inl* = inner nuclear layer; *ipl* = inner plexiform layer; *onl* = outer nuclear layer; *opl* = outer plexiform layer; *vz* = ventricular zone.

Fig. 3.

Effects of CNTF on retinal cell proliferation. Quantifications of BrdU incorporation in the presence (gray) or absence (white) of CNTF in vitro are shown. **A** Percentages of BrdU⁺ cells among total cells in P0 and P3 monolayer cultures at various times with or without CNTF (10 ng/ml). BrdU (20 μ M) labeling was for 12 h before harvesting. Results are presented as the mean $\pm$ SEM of 3 independent experiments ($n = 3$). **B** Percentages of BrdU⁺ cells among total cells in P0 retinal explants cultured for various DIV with or without CNTF (100 ng/ml). BrdU (20 μ M) labeling was for 24 h before harvesting. Results are presented as the mean $\pm$ SEM of 3 independent experiments ($n = 3$) for P0-P1 and P0-P3, and 6 independent experiments ($n = 6$) for P0-P6. The asterisks ** indicate p values <0.01 when compared to nontreated controls.

Fig. 4.

CNTF promotes Müller glial cell fate from the retinal progenitor pool. Quantifications of Müller cell marker and BrdU double-positive cells among BrdU⁺ cells. **A** Percentages of BrdU⁺ GFAP⁺ cells (gray) and BrdU⁺ CRALBP⁺ cells (white) among total BrdU⁺ cells labeled with BrdU (20 μ M) at P0 for 12 h and then cultured as explants in fresh medium in the presence or absence of CNTF (10 ng/ml) for 5.5 DIV. Results are presented as the mean $\pm$ SEM of 3 independent experiments (n = 3). **B** Percentages of BrdU⁺ GFAP⁺ cells among total BrdU⁺ cells in retinal explants cultured in the presence or absence of CNTF (100 ng/ml) for 6 DIV and labeled with BrdU 20 μ M) at P5 for 24 h. Results are presented as the mean $\pm$ SEM of 6 independent experiments (n = 6). The asterisks * and ** indicate p values <0.05 and <0.01 when compared to nontreated samples, respectively.

Fig. 5.

Effects of signaling pathway-specific perturbation on CNTF-induced Müller glial genesis.

A, B Percentages of GFAP⁺ cells (**A**) and cyclin D3⁺ cells (**B**) among total cells in P0 retinal explants cultured for 6 DIV in the presence or absence of CNTF (100 ng/ml) and the MEK inhibitor U0126 (10 μ M). Results are presented as the mean $\pm$ SEM of 6 independent experiments (n = 6). **C** Western blots showing inhibition of CNTF-induced STAT3 phosphorylation by AG490 in monolayer retinal cultures. AG490 was added to cells 1 h prior to 15 min of CNTF (10 ng/ml) stimulation. Top and bottom panels show phospho-proteins and total proteins, respectively. **D** Percentages of Müller markers GFAP (gray) and CRALBP (white) in P0 retinal explants cultured for 6 DIV in the presence or absence of CNTF (10 ng/ml) and Jak inhibitor AG490 (10 μ M). Results are expressed as the mean $\pm$ SEM of 3 independent experiments (n = 3). **E** Percentages of Müller marker cyclin D3⁺ cells among GFP⁺ cells in transfected P0 retinal explants cultured for 6 DIV without exogenous ligands. Results are expressed as the mean $\pm$ SEM of 3 independent experiments (n = 3). The asterisks * and ** indicate p values <0.05 and <0.01 between conditions, respectively.

Fig. 6. Effects of anti-EGF antibody on CNTF-dependent Müller glia production. Percentages of GFAP⁺ (**A**) and cyclin D3⁺ (**B**) cells among total cells in P0 retinal explants cultured for 6 DIV in the presence and absence of CNTF (100 ng/ml) and anti-EGF antibody (10 µg/ml). Results are presented as the mean ± SEM of 6 independent experiments (n = 6). The asterisks ** indicate p values <0.01 between conditions.

Table 1.

Summary of antibodies

Name ¹	Type ²	Dilution ratios ³	Source/reference
<i>Primary antibodies</i>			
p-ERK1/2 (Thr202/Tyr204)	rAb	1:200; 1:1,000 (w)	Cell Signaling Inc.
p-ERK1/2 (Thr202/Tyr204, E10)	mAb	1:100	Cell Signaling Inc.
ERK2 (C-14)	rAb	1:1,000 (w)	Santa Cruz Biotech.
p-STAT3 (Tyr705)	rAb	1:100; 1:1,000 (w)	Cell Signaling Inc.
STAT3 (K-15)	rAb	1:1,000 (w)	Santa Cruz Biotech.
BrdU	mAb	1:5	Amersham
PCNA (PC10)	mAb	1:500	Sigma
CRALBP	rAb	1:200	De Leeuw et al., 1990
Cyclin D3	mAb	1:100	Cell Signaling Inc.
GFAP	mAb	1:400	Chemicon
GFAP	rAb	1:400	Sigma
GS	mAb	1:50	Chemicon
EGF	rAb	10 µg/ml (b)	Upstate
<i>Secondary antibodies</i>			
Texas red-conjugated	dAb	1:200	Jackson Imm. Res. Lab.
Alexa 488- or 594-conjugated	gAb	1:400	Molecular Probes
HRP-conjugated	gAb	1:1,000 (w)	Amersham

¹For full names of the antigens, see the text. Phospho-proteins are abbreviated as 'p-'

²The antibody-producing species are indicated by lower case letters preceding the 'Ab'. r = Rabbit; m = mouse; d = donkey; g = goat.

³Unless noted, the dilution ratios indicated are for immunostaining. Antibody dilutions used for Western blots or activity blocking are followed by (w) and (b), respectively.