Neuron, Vol. 48, 253–265, October 20, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.neuron.2005.08.037

Evidence that Embryonic Neurons Regulate the Onset of Cortical Gliogenesis via Cardiotrophin-1

Fanie Barnabé-Heider,[1,5] Julie A. Wasylnka,[1,2] Karl J.L. Fernandes,[1,2] Christian Porsche,[6] Michael Sendtner,[6] David R. Kaplan,[2,3,5] and Freda D. Miller[1,3,4,5,*]

[1]Developmental Biology
[2]Cancer Research
Hospital for Sick Children
[3]Department of Molecular and Medical Genetics
[4]Department of Physiology
University of Toronto
Toronto M5G 1X8
Canada
[5]Department of Neurology and Neurosurgery
McGill University
Montreal H3A 2B4
Canada
[6]Institute of Clinical Neurobiology
University of Wuerzburg
Wuerzburg 97080
Germany

Summary

Precursor cells of the embryonic cortex sequentially generate neurons and then glial cells, but the mechanisms regulating this neurogenic-to-gliogenic transition are unclear. Using cortical precursor cultures, which temporally mimic this in vivo differentiation pattern, we demonstrate that cortical neurons synthesize and secrete the neurotrophic cytokine cardiotrophin-1, which activates the gp130-JAK-STAT pathway and is essential for the timed genesis of astrocytes in vitro. Our data indicate that a similar phenomenon also occurs in vivo. In utero electroporation of neurotrophic cytokines in the environment of embryonic cortical precursors causes premature gliogenesis, while acute perturbation of gp130 in cortical precursors delays the normal timed appearance of astrocytes. Moreover, the neonatal *cardiotrophin-1*$^{-/-}$ cortex contains fewer astrocytes. Together, these results describe a neural feedback mechanism; newly born neurons produce cardiotrophin-1, which instructs multipotent cortical precursors to generate astrocytes, thereby ensuring that gliogenesis does not occur until neurogenesis is largely complete.

Introduction

Development of the cerebral cortex is achieved through a common pool of precursor cells that sequentially generate neurons and glial cells. Remarkably, this precisely timed process can be recapitulated in vitro with isolated embryonic cortical stem cells (Qian et al., 2000), suggesting that cortical precursors are biased initially to generate neurons and that an undefined mechanism instructs them to generate astrocytes at later time points. One potential explanation for this timing mechanism is a cell-intrinsic one where, over time, precursor cells themselves change so they are less biased to make neurons and more likely to make glial cells. Such a change could occur, for example, as a consequence of dilution of a neurogenic factor(s) such as a transcription factor (Nieto et al., 2001; Sun et al., 2001; Ménard et al., 2002; He et al., 2005), by expression of a receptor such as EGFR (Burrows et al., 1997; Sun et al., 2005), or by demethylation of astrocyte-specific gene promoters (Takizawa et al., 2001). Another, not mutually exclusive, explanation is that cortical precursor cells are prematurely competent to generate astrocytes, but they require an extrinsic gliogenic signal that is not present in their environment until later in embryogenesis. Indeed, several exogenous growth factors have been shown to be potent gliogenic factors (reviewed in Sauvageot and Stiles, 2002). In particular, the interleukin-6 (IL-6) family of neurotrophic cytokines, which includes leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1), induces premature astrocyte formation in cultured cortical precursors via a gp130-JAK-STAT pathway (Bonni et al., 1997; Rajan and McKay, 1998; Ochiai et al., 2001), and targeted ablation of the genes encoding either gp130 or its coreceptor LIFR perturbs astrocyte formation in vivo (Ware et al., 1995; Koblar et al., 1998; Nakashima et al., 1999a).

While these latter studies indicate that cytokine signaling is important, the finding that the appropriate timing of astrogenesis is mimicked in isolated clones of precursor cells seems to argue that a cell-intrinsic mechanism regulates the onset of gliogenesis (Qian et al., 2000). However, since such clones contain both precursors and newly born neurons, a second hypothesis is that newly born neurons secrete gliogenic factors that feed back to instruct multipotent precursors to generate astrocytes. Here, we describe data that support this hypothesis. Specifically, we demonstrate that newly born neurons secrete the gliogenic cytokine CT-1, which then instructs cortical precursors to generate astrocytes at the appropriate time and in the appropriate number. These findings support a model where cortical precursors are competent to generate astrocytes early during cortical development, but only later in embryogenesis, when levels of neuron-secreted CT-1 are sufficiently high, do these precursors receive the necessary gliogenic signal to initiate astrocyte formation, thereby ensuring that gliogenesis does not commence until neurogenesis is largely complete.

Results

Mixed Cortical Neuron and Precursor Cultures Induce Premature Astrocyte Formation from Early Cortical Precursors

To study mechanisms responsible for the sequential generation of neurons and glia, we examined primary murine (embryonic day 13) E13 cortical precursor cells (Toma et al., 2000; Ménard et al., 2002; Barnabé-Heider and Miller, 2003), a system that we have previously

*Correspondence: fredam@sickkids.ca

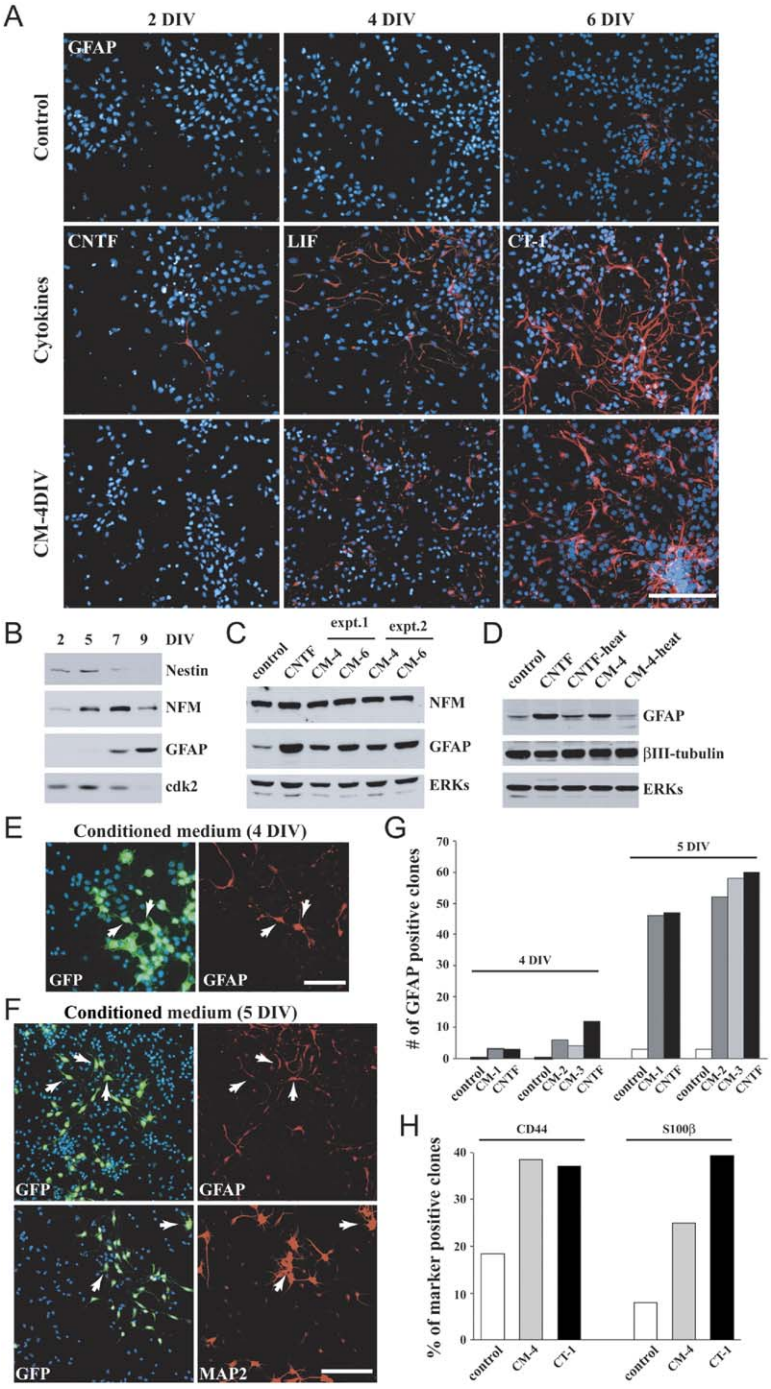

Figure 1. Conditioned Medium from Mixed Neuron and Cortical Precursor Cultures Instructs Multipotent Cortical Precursors to Generate Astrocytes Prematurely

(A) GFAP immunofluorescence (red) in precursors cultured for 2, 4, and 6 DIV in 50 ng/ml CNTF, LIF, or CT-1 or 4 DIV CM. Scale bar, 100 μm.

(B) Western blots of precursors after 2–9 DIV for nestin, cdk2, NFM, and GFAP.

(C and D) Western blots of precursors cultured for 6 days in CNTF or in two sets of 4 DIV or 6 DIV CM (expt. 1 and expt. 2) (C) or in a third batch of 4 DIV CM that was or was not heat denatured (CM-4 and CM-4-heat) (D). Blots were probed for NFM, βIII-tubulin, and GFAP and reprobed for total ERK levels as a loading control.

(E–H) Clonal analysis demonstrates that conditioned medium recruits multipotent cortical precursors to generate astrocytes. (E and F) E13 EYFP-tagged precursors were cultured at clonal density with age-matched CD1 precursors for 4 (E) or 5 (F) days in CM and immunostained for EYFP (GFP, green) and GFAP or MAP2 (both red). Photomicrographs show individual clones, and arrows show double-labeled cells. Scale bar, 50 and 100 μm in (E) and (F), respectively. (G and H) Number of clones containing cells expressing GFAP (G), CD44 (H), or S100β (H) in cultures grown in CNTF, CT-1, or CM versus control medium. CM1-4 refers to four independent batches of CM. In (A), (E), and (F), cells were counterstained with Hoechst 33258 (blue) to show all cells in the field.

characterized. Upon plating, these cortical precursors were virtually all dividing, nestin-positive cells that sequentially generated neurons and then glia, as visualized by immunocytochemistry and Western blots for neurofilament-M (NFM; Figure 1B), glial fibrillary acidic protein (GFAP; Figures 1A and 1B), galactocerebrosidase (GalC), and CNPase (Figure S1A in the Supplemental Data available with this article online). Neurons were first observed after 1 day in vitro (DIV) (Figure 1B; data not shown), while astrocytes and oligodendrocytes were first seen at 5–6 DIV (Figures 1A and 1B; Figure S1A). The increase in differentiated cells was accompanied by a depletion of precursors, as shown by a reduction in expression of nestin and the proliferating cell marker cyclin-dependent kinase 2 (cdk2; Figure 1B).

One explanation for why gliogenesis follows neurogenesis in these cultures is that newly born neurons produce a gliogenic factor. To test this possibility, we exposed newly plated cortical precursors to medium conditioned by 4-day-old cortical precursor cultures (4 DIV conditioned medium [CM]), which contain only neurons and precursors (Figures 1A and 1B). As a positive control, we used the known gliogenic cytokines CNTF, LIF, and CT-1 (Bonni et al., 1997; Rajan and McKay,

1998; Ochiai et al., 2001). Immunocytochemistry for GFAP revealed that at 4 DIV no astrocytes were present in controls, but that cultures treated with CM or any of the three cytokines contained astrocytes, the numbers of which increased dramatically by 6 DIV (Figure 1A). We extended these results by examining CD44 and S100β, which we confirmed are expressed in astrocyte precursors/early astrocytes and glial precursors/early astrocytes, respectively, in these cultures (Figures S1B and S1C; Burrows et al., 1997; Liu et al., 2004). As predicted, CD44- and S100β-positive cells were detected earlier than those expressing GFAP (Figures S2A and S2B), and at 4 DIV, CM increased their numbers by approximately 2-fold (Figures S2A and S2B; Figures 4J and 4K). Western blots at 6 DIV confirmed these findings and showed that levels of GFAP, but not NFM or βIII-tubulin, were increased by CM (Figures 1C and 1D). Since the gliogenic effect of the CM was inhibited by heat denaturation (Figure 1D), we conclude that it is likely due to a secreted protein.

To ask whether this premature gliogenesis reflected an increase in the number of cortical precursors that generated astrocytes, we performed clonal analysis. Since survival of cortical precursors is density dependent (Barnabé-Heider and Miller, 2003), we seeded E13 EYFP-tagged transgenic cortical precursors at clonal density on untagged E13 precursors. These cultures were grown 4 or 5 days with or without 4 DIV CM or CNTF; over this time period, single EYFP-positive cells proliferated to generate clones (Figures 1E and 1F). Immunocytochemistry revealed that, in control cocultures at 4 DIV, most clones contained cells expressing MAP2 and nestin, but no clones contained GFAP-positive astrocytes (data not shown; Figure 1G). By 5 DIV, a very small number of clones contained at least one GFAP-positive cell (Figure 1G). In contrast, when cells were cultured in 4 DIV CM, some clones contained GFAP-positive cells at day 4 (Figures 1E and 1G), while many more did on day 5 (Figures 1F and 1G). To ask whether CM enhances genesis of astrocytes versus enhancing differentiation of previously determined astrocyte precursors, we also examined CD44 and S100β (Figure 1H). At 4 DIV, 2 to 3 times as many clones contained CD44- or S100β-positive cells when exposed to CM (Figure 1H), an effect quantitatively similar to that seen in cultures exposed to CNTF or CT-1 (Figures 1G and 1H). In contrast, neither CM nor CNTF affected the number of clones containing MAP2-positive neurons or nestin-positive precursors (Figure 1F; data not shown). Thus, a factor(s) secreted by the mixed precursor/neuron cultures directs cortical precursors to generate astrocytes.

Purified Embryonic Cortical Neurons Secrete Factors that Are Sufficient to Direct Early Cortical Precursors to Generate Astrocytes

To identify the source of the gliogenic factors contained in CM, we isolated purified embryonic cortical neurons, using the K6 line of Tα1:nlacZ transgenic mice, which expresses nuclear β-galactosidase from the neuron-specific Tα1 α-tubulin promoter (Gloster et al., 1999). E15-17 transgenic cortical cells were labeled with the vital dye fluorescein di-β-D-galactopyranoside (FDG), sorted by FACS, and cultured for 5 days. Immunocytochemistry demonstrated that 99.5% of the cells expressed βIII-tubulin, the vast majority of which also expressed MAP2 (Figure 2A), while no cells expressed glial markers (data not shown). CM was collected from these purified neurons and applied to newly isolated cortical precursors for 5 days. Immunocytochemistry revealed that the control cultures contained many MAP2-positive neurons with only the rare GFAP-positive astrocyte, while the cultures grown in neuron-derived CM contained both neurons and astrocytes (Figure 2B). Quantitation revealed that pure neuron CM was similar to exogenous CNTF in its ability to promote premature astrocyte formation (Figure 2C).

Developing Cortical Neurons Express Neurotrophic Cytokines that Activate Downstream Cytokine Signaling Pathways in Cortical Precursors

CNTF, CT-1, and LIF promote astrocyte differentiation from cortical precursors via activation of the JAK-STAT pathway (Bonni et al., 1997; Rajan and McKay, 1998; Nakashima et al., 1999b; Ochiai et al., 2001). To determine whether the cortical neuron gliogenic factor(s) was a cytokine, we performed semiquantitative RT-PCR. This analysis revealed that the mRNAs for CNTF, LIF, and CT-1 were not expressed in freshly isolated precursors but were detectable in mixed precursor/neuron cultures at 6 DIV, with the highest levels in cortical neurons isolated at E15 and cultured for 5 DIV (Figure 2D). We also confirmed using RT-PCR and Western blots that the gp130 receptor (required for actions of all the cytokines) and LIFR (required for actions of LIF, CNTF, and CT-1) were expressed in cortical precursors from 0 to 8 DIV (Figure 2E; data not shown).

To confirm that cortical neurons produce cytokines, we asked whether CM induced the JAK-STAT pathway. Initially, we characterized the basal level of JAK-STAT activation in cortical precursor cultures. Western blots and immunocytochemistry revealed that JAK1, JAK2, and STAT3 were all expressed from 1 to 6 DIV (Figure 2G), that STAT3 was enriched in precursors relative to newly born neurons (Figure 2F), and that the phosphorylated, activated forms of JAKs 1 and 2 increased at 4–6 DIV (Figure 2G) coincident with the predicted onset of endogenous gliogenic signaling. To ask whether CM activated this same pathway, 4 DIV precursors were washed, exposed to CM for 15 min, and analyzed by Western blots. Results demonstrated that CM from either purified neurons or 4 DIV precursors induced activation of JAK2 and STAT3 (Figure 2H), as did CNTF. CM and CNTF also acutely activated a second cytokine signaling pathway, the MEK-ERK pathway, as assayed with a phospho-ERK antibody (Figure 2H). Thus, newly born cortical neurons secrete cytokines that activate appropriate signaling pathways in multipotent cortical precursors.

Cytokine Signaling through the JAK-STAT Pathway, but Not through MEK, Is Necessary for Cortical Neurons to Induce Premature Gliogenesis

Cytokines are thought to mediate gliogenesis from cortical precursors by activation of the JAK-STAT but not the MEK-ERK pathway, which is instead thought to promote neurogenesis in cortical precursors (Ménard et al., 2002; Barnabé-Heider and Miller, 2003). To assess the

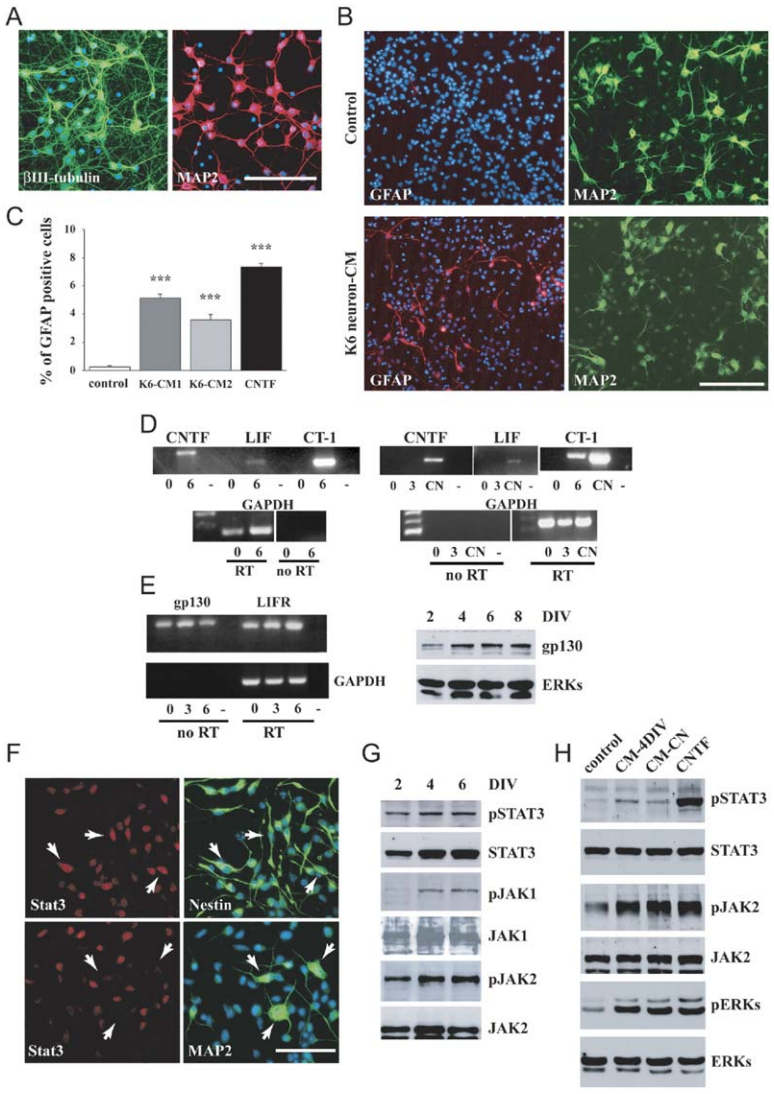

Figure 2. Embryonic Cortical Neurons Secrete Cytokines that Direct Multipotent Cortical Precursors to Generate Astrocytes

(A) Immunostaining for βIII-tubulin (green) or MAP2 (red) in E15 K6 cortical neurons purified by FACS and cultured 5 days. Scale bar, 100 μm.

(B) Immunostaining for GFAP (red) and MAP2 (green) in precursors cultured 5 days without (top panels) or with (bottom panels) neuron-derived CM. Scale bar, 100 μm.

(C) Percentage of GFAP-positive cells in experiments performed as in (B) using two independent preparations of neuron-derived CM (K6-CM1-2) or CNTF. ***$p < 0.001$ relative to control medium (ANOVA). Error bars indicate SEM.

(D–H) Developing cortical neurons express cytokines, and neuron-conditioned medium activates cytokine signaling in cortical precursor cells. (D) RT-PCRs for CNTF, LIF, and CT-1 mRNAs in freshly dissociated precursors (0), after culturing 3 or 6 DIV, or from developing cortical neurons (CN). GAPDH was used as a loading and genomic DNA contamination control. (E) RT-PCRs (left) and Western blots (right) for gp130 and LIFR in precursors from 0 to 8 DIV. (F) Immunostaining for STAT3 (red) and nestin (green; top panels) or MAP2 (green; bottom panels) in 4 DIV precursors. Arrows denote the same cells. Scale bar, 50 μm. (G) Western blots of precursors at 2 to 6 DIV for STAT3, JAK1, and JAK2 and their phosphorylated, activated forms (pSTAT3, pJAK1, and pJAK2). (H) Western blots for phosphorylated STAT3, JAK2, or ERKs (pSTAT3, pJAK2, pERKs) in 4 DIV precursors stimulated 15 min with CM from 4 DIV precursors (CM-4DIV) or cortical neurons (CM-CN), or with CNTF. Blots were probed for total STAT3, JAK2, or ERKs as loading controls. In (A), (B), and (F), cells were counterstained with Hoechst 33258 (blue).

role of these two pathways in CM-mediated gliogenesis, we used AG490 and PD98059, which inhibit JAK and MEK, respectively. To ascertain the specificity and efficacy of these inhibitors, we cultured precursors for 3 days, at which point the peak of neurogenesis is over, and then added CNTF or CM with or without 50 μM PD98059 or 5 μM AG490 for 2 additional days. As predicted, PD98059 inhibited long-term ERK phosphorylation with no effect on the JAK-STAT pathway (Figure 3C; data not shown), while AG490 had the converse effect (Figure 3D). We then asked whether either of these two inhibitors affected gliogenesis. Immunocytochemistry and Western blots revealed that AG490 but not PD98059 decreased the gliogenic effect of CNTF and completely abolished CM-induced astrocyte formation (Figures 3A and 3C–3E). Neither inhibitor affected the numbers of neurons (Figures 3C–3E), likely because neurogenesis is largely complete by the time the inhibitors are added. Total cell numbers were also similar in all conditions (CM-treated cultures had 1028 ± 135 cells per field with DMSO, 975 ± 88 with AG490, and 1017 ± 117 with PD98059), indicating that these inhibitors did not affect survival or proliferation. Moreover, when AG490 was applied at 7 DIV, after astrocytes were already born, it had little or no effect on astrocyte number (Figure 3B), arguing that it specifically affected gliogenesis.

To confirm that the JAK-STAT pathway was essential for CM-induced gliogenesis, we inhibited it genetically, using siRNA for STAT3. To demonstrate the efficacy of the STAT3 siRNA vector, we cotransfected it with an EGFP expression plasmid into NIH-3T3 cells. Although transfection efficiency was only 30%–50%, Western blots demonstrated that STAT3 levels were decreased at time points from 2 to 6 days posttransfection (Figure 4A; data not shown). We then asked whether STAT3 siRNA knockdown would inhibit gliogenesis; precursors were cotransfected immediately after plating, cultured in 4 DIV CM or CNTF, and analyzed after 5 days. The efficacy of the siRNA was demonstrated by immunostaining cells for GFP and STAT3 (Figure 4B); 85% of GFP-positive control cells versus 29% of STAT3 siRNA-transfected cells were immunoreactive for STAT3. Immunostaining of these cultures for GFP and GFAP or MAP2 revealed that STAT3 siRNA reproducibly decreased by 2- to 3-fold the number of astrocytes

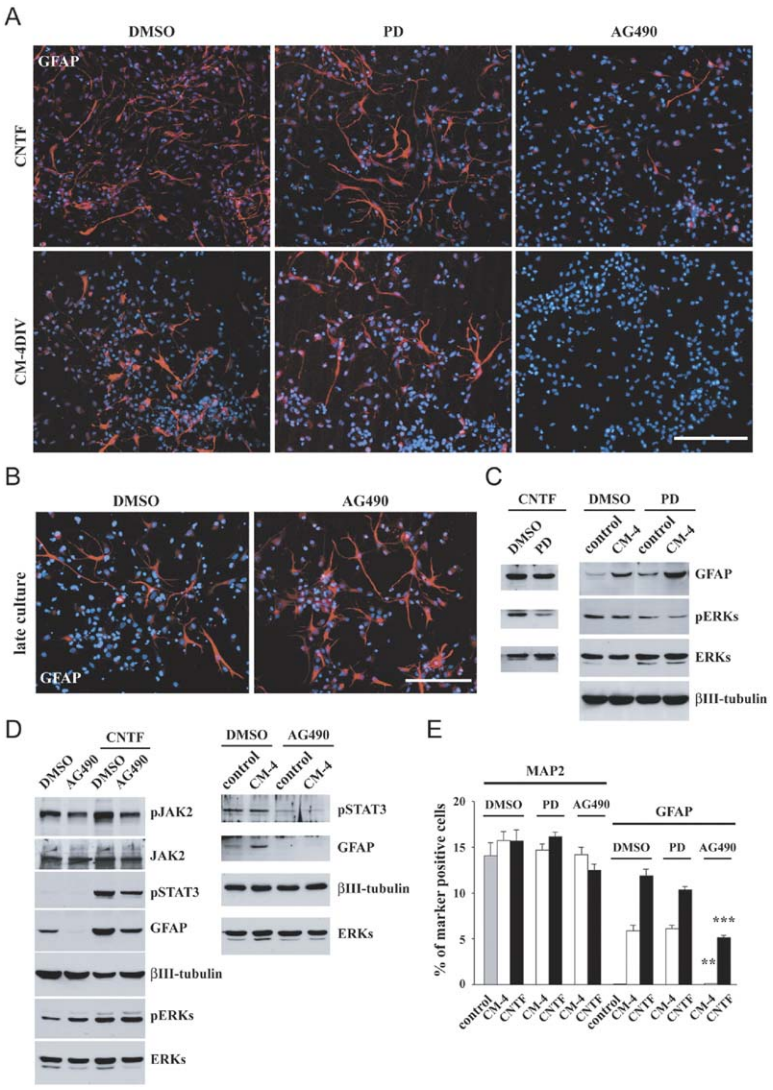

Figure 3. Inhibition of the JAK-STAT, but Not the MEK Pathway, Prevents Conditioned Medium-Induced Astrocyte Formation

(A) GFAP immunostaining (red) in precursors cultured 3 days and treated for 2 additional days with 4 DIV CM or CNTF with or without 5 μM AG490, 50 μM PD98059 (PD), or DMSO.

(B) GFAP immunostaining (red) in precursors cultured 7 days and treated with AG490, as in (A). In (A) and (B), cells were counterstained with Hoechst 33258 (blue). Scale bars, 100 μm.

(C and D) Western blots of precursors treated as in (A) with 50 μM PD98059 (C) or 5 μM AG490 (D). Blots were probed with antibodies to total or phosphorylated ERKs, STAT3, and JAK2, or to GFAP and βIII-tubulin.

(E) Percentage of GFAP- or MAP2-positive cells in one representative experiment of three experiments that gave similar results to that shown in (A). ***$p < 0.001$, **$p < 0.01$ relative to DMSO controls (ANOVA). Error bars indicate SEM.

generated in either CM or CNTF (Figures 4C and 4D). Interestingly, STAT3 knockdown also increased the number of neurons by approximately 2-fold (Figures 4C and 4D), indicating that the JAK-STAT pathway both enhances gliogenesis and inhibits neurogenesis.

These findings indicate that newly born neurons secrete factors that promote gliogenesis via the JAK-STAT pathway. To determine whether these factors are cytokines, we decreased levels of the cytokine receptor gp130 on cortical precursors using a mixed pool of siRNA oligonucleotides. Western blots showed that, when transfected into NIH-3T3 cells, these oligonucleotides reduced gp130 levels relative to cells transfected with a scrambled oligonucleotide (Figure 4E), in spite of a transfection efficiency of only 30%–50%. We then cotransfected cortical precursors with GFP and gp130 siRNA at 1 day after plating and maintained them for 4 additional days in 4 DIV CM or CNTF. This protocol effectively reduced gp130 levels (Figure 4F); 82% of control cells versus 26% of gp130 siRNA-transfected cells were immunoreactive for gp130. Immunostaining of these cultures for GFAP, S100β, and CD44 revealed that, relative to the scrambled oligonucleotide, gp130 knockdown dramatically reduced the number of cells expressing these astrocyte precursor/astrocyte markers in response to CM (Figures 4G–4K). The endogenous gliogenesis that occurs in control cultures was also highly reduced (Figures 4J–4K). Interestingly, gp130 knockdown increased the number of MAP2-positive neurons by approximately 2-fold (Figure 4G). Thus, the gliogenic factor(s) in CM is a cytokine(s), and a reduction in gp130 signaling in cortical precursors inhibits gliogenesis and promotes neurogenesis.

Cortical Precursor Cells in the Early Embryonic Ventricular/Subventricular Zone Are Competent to Generate Astrocytes in Response to Premature Neurotrophic Cytokine Expression

Our findings suggest that cortical precursors are competent to make astrocytes earlier in development but that they do not do so because of low cytokine levels in their environment. To test this idea, we expressed the cytokine CNTF in the embryonic telencephalic VZ/SVZ by performing in utero electroporation with a plasmid encoding a myc-tagged, secreted form of CNTF (Jiang et al., 2003). As predicted, transfection of this

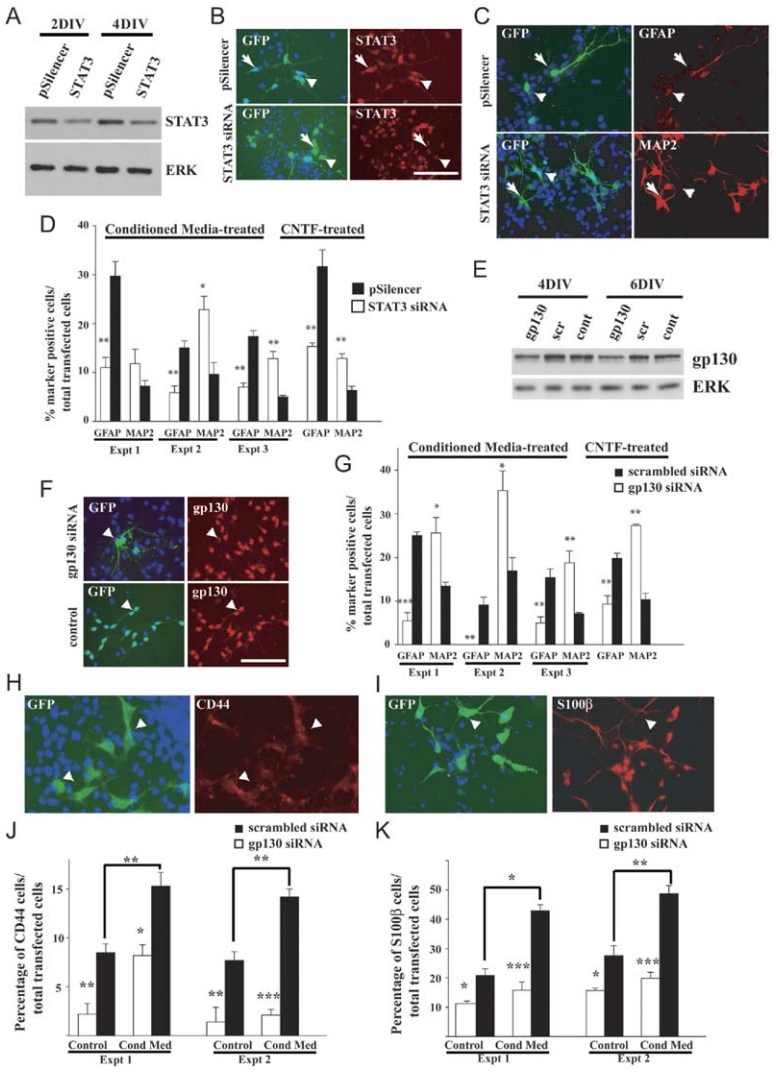

Figure 4. A Knockdown of Cytokine Signaling Using siRNA for STAT3 or gp130 Inhibits Conditioned Medium-Directed Astrogenesis

(A) Western blot for STAT3 in NIH 3T3 cells transfected for 2 or 4 days with empty control vector (pSilencer) or STAT3 siRNA. The blot was reprobed for ERK.

(B) Immunostaining for GFP (green) and STAT3 (red) in precursors cotransfected 5 days earlier with GFP and either STAT3 siRNA (bottom panels) or empty pSilencer vector (top panels). Arrows and arrowheads represent transfected cells.

(C) Immunostaining for GFP (green) and GFAP (red; top panels) or MAP2 (red; bottom panels) in precursors cotransfected as in (B) and treated with 4 DIV CM. Arrows and arrowheads denote transfected cells that do or do not express the relevant marker protein, respectively.

(D) Quantitation of experiments similar to those shown in (C), with CNTF treatment as a positive control. For each condition, at least 300 cells were counted in each of three independent experiments (Expt. 1, Expt. 2, and Expt. 3). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ relative to vector-transfected cells (Student's t test). Error bars indicate SEM.

(E) Western blots of NIH 3T3 cells transfected with gp130 or scrambled (scr) siRNA oligonucleotides for 4 or 6 days. Untransfected cells (cont) were run as a control. The blot was reprobed for total ERKs.

(F) Immunostaining for GFP (green) and gp130 (red) in precursors cotransfected 5 days earlier with the GFP plasmid and either gp130 siRNA oligonucleotides (top panels) or the scrambled oligonucleotide (control, bottom panels). Arrowheads represent transfected cells.

(G) Quantitation of cotransfected precursors treated with 4 DIV CM or CNTF for 5 days and immunostained for GFP and GFAP or GFP and MAP2. For each condition, at least 300 cells were counted in each of three independent experiments (Expt. 1, Expt. 2, and Expt. 3). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ relative to the scrambled oligonucleotide-transfected cells (Student's t test). Error bars indicate SEM.

(H and I) Immunostaining for GFP (green) and CD44 (H) or S100β (I) (both red) in precursors treated as in (G). Arrowheads denote the same cells.

(J and K) Quantitation of experiments similar to those shown in (H) and (I). For each condition, at least 300 cells were counted in each of two independent experiments (Expt. 1 and Expt. 2). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ relative to the scrambled oligonucleotide-transfected cells, or for the control groups joined by black bars, between control and CM-treated cells (Student's t test). Error bars indicate SEM. In (B), (C), (F), (H), and (I), cells were counterstained with Hoechst 33258 (blue); scale bars, 100 μm.

plasmid into COS cells led to secretion of CNTF into the medium (Figure S3C). Moreover, when cortical precursors were transfected with this plasmid, at 5 DIV more than 30% of the cells expressed GFAP, and the JAK-STAT pathway was robustly activated (Figures S3A–S3C). Importantly, many nontransfected cells were GFAP positive (Figure S3A), as would be predicted if CNTF was secreted by the transfected cells.

We then performed in utero electroporation; plasmids encoding a nuclear-targeted GFP and CNTF or empty vector were coelectroporated following injection into the E15 lateral ventricles, embryos were reimplanted, and cortices were analyzed 1–3 days later. One day following electroporation, only cells in the VZ/SVZ of the telencephalon were transfected (data not shown), as previously reported (Ohtsuka et al., 1999). Three days following electroporation, at E18, many cells were still in the VZ/SVZ, but many had also migrated into the cortical plate (Figure 5A). Immunocytochemistry demonstrated that virtually no GFAP-positive cells were detected in the control-transfected E18 cortex (Figure 5A, left panels) but that many astrocytes were present in the CNTF-transfected cortical VZ/SVZ (Figure 5A, right panels). Confocal microscopy revealed that approximately 20% of GFP-CNTF-positive cells in the SVZ adopted an astrocytic fate (Figures 5B and 5C) and that many nontransfected cells also expressed GFAP (Figure 5B), as was seen in the culture experiments (Figure S3A).

To ask how early cortical precursors were competent to generate astrocytes in vivo, we performed the same electroporation experiment at the earliest feasible time point, E13.5. Immunocytochemical analysis of these brains 2.5 days later, at E16, revealed abundant GFAP-positive cells within the VZ/SVZ of CNTF-transfected but not control-transfected animals (Figure 5F).

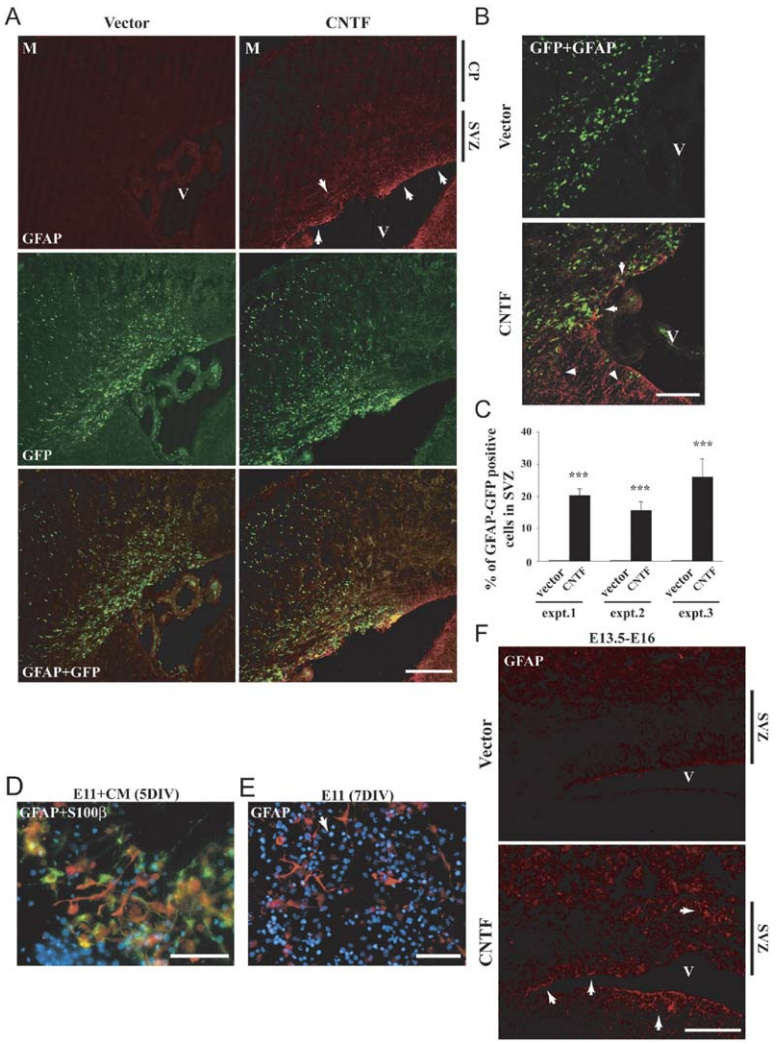

Figure 5. Embryonic Cortical Precursors Are Competent to Respond to CNTF with Premature Gliogenesis In Vivo

(A–C) In utero electroporations of precursor cells in the E15 telencephalic VZ/SVZ with plasmids encoding GFP and either CNTF or empty vector. Three days after electroporation, coronal telencephalic sections were immunostained for GFP and GFAP. (A) E18 cortical sections from embryos transfected with the empty vector (left panels) or with CNTF plasmid (right panels) and immunostained for GFAP (red, top) and GFP (green, middle; bottom shows merged images). V, ventricle; SVZ, subventricular zone; CP, cortical plate; M, meninges. Scale bar, 200 μm. (B) Confocal microscopic analysis of GFP (green) and GFAP (red) expression in the SVZ of sections similar to those in (A). Scale bar, 100 μm. Arrows denote transfected GFAP-positive cells, and arrowheads denote nontransfected GFAP-positive cells. (C) Quantitation of sections similar to those in (B). Transfected cells within the SVZ of three littermate pairs, four sections per embryo, were counted. ***$p < 0.001$ relative to vector-transfected sections (Student's t test). Error bars indicate SEM.

(D and E) Immunostaining for GFAP (red) and/or S100β (green) in E11.5 precursors cultured for 5 DIV in CM (D) or 7 DIV in control medium (E). Cells were counterstained with Hoechst 33258. Scale bar, 50 μm.

(F) E16 cortical sections that were electroporated at E13.5 with either an empty vector or a CNTF plasmid and immunostained for GFAP (red). Arrows indicate regions of GFAP immunoreactivity. Scale bar, 100 μm.

Immunohistochemistry for S100β revealed a similar enhancement in the CNTF-transfected VZ/SVZ (data not shown), arguing that CNTF promoted the premature genesis of astrocytes, and not just their differentiation.

We also asked how early cortical precursors were competent to respond to the cytokine(s) in CM; precursors were cultured from the E11.5 cortex and exposed to CM or CNTF. Immunocytochemical analysis revealed that at 5 DIV (the earliest time point examined), many S100β- and GFAP-positive astrocytes were present under both of these conditions (Figure 5D), while none were present in control cultures until 7 DIV (Figure 5E). Thus, early cortical precursors are competent to respond to cytokines both in culture and in vivo, arguing that E13–E15 cortical precursor cells do not normally produce astrocytes because of the limited availability of gliogenic factors in their environment.

The Appropriate Onset of Gliogenesis In Vivo Requires Cytokine Signaling in Cortical Precursor Cells

While these findings argue that cortical precursors are competent to respond to cytokines during embryogenesis, they do not demonstrate that cytokines regulate the appropriate onset of gliogenesis. To address this issue, we acutely perturbed cytokine signaling in cortical precursors using gp130 siRNA. As previously reported (Calegari et al., 2002), plasmids and siRNA oligonucleotides can be efficiently coelectroporated into the developing cortex and remain stably expressed (data not shown). We therefore coelectroporated a GFP plasmid with either scrambled or gp130 siRNA oligonucleotides into the E14/15 telencephalon and analyzed the cortices at postnatal day 3 (P3) or P4. Immunocytochemistry for GFP and GFAP or NeuN revealed that, in control animals, almost all of the GFAP-positive cells were localized to the SVZ (Figure 6A), while the vast majority of NeuN-positive neurons were in the cortical plate (Figure 6D). Confocal analysis demonstrated that, at P3, GFAP was expressed in 5%–10% of the GFP-positive control-transfected cells, but in virtually none of the gp130 siRNA-transfected cells (Figures 6B and 6C). By P4, 20%–30% of the control, GFP-positive cells expressed GFAP, while only approximately 5% of the gp130 siRNA-transfected cells were GFAP positive (Figures 6B and 6C). In contrast, gp130 siRNA knockdown led to a small but significant increase in the number of transfected neurons in the cortical plate (Figures 6D and 6E), consistent with the culture data. Thus, the appropriate onset of astrocyte formation requires the

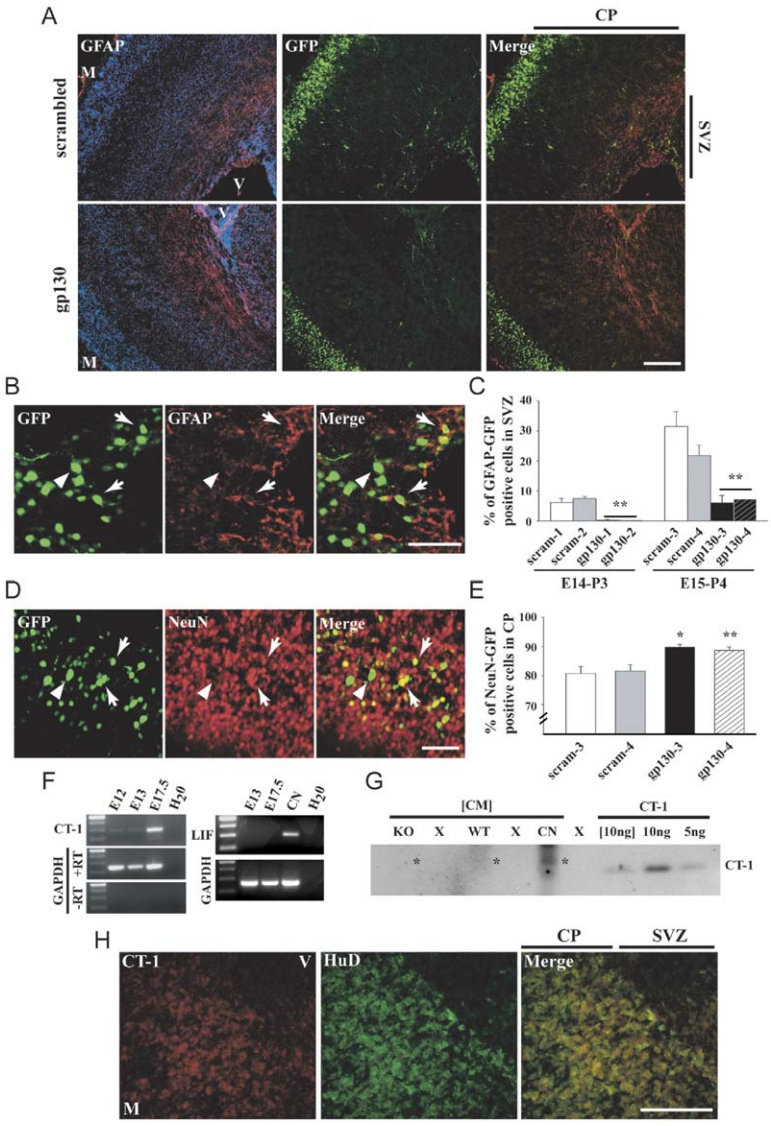

Figure 6. Cytokine Signaling in Cortical Precursors Is Essential for the Appropriate Onset of Astrocyte Formation In Vivo

gp130 siRNA or scrambled oligonucleotides were coelectroporated with a control GFP plasmid into E14/15 embryos, and neonatal cortical sections were then immunostained for GFP and GFAP (A–C) or GFP and NeuN (D and E). (A) Immunostaining for GFAP (red, left; also shown is the Hoechst 33258 counterstain in blue), GFP (green, middle; right panels are the merged images) on P4 cortical sections from animals transfected with either scrambled (top panels) or gp130 siRNAs (bottom panels). V, ventricle; SVZ, subventricular zone; CP, cortical plate; M, meninges. Scale bar, 200 μm. (B and D) Confocal microscopic analysis of GFP (green) and GFAP (B) or NeuN (D) (both red) in the P4 SVZ (B) or CP (D) of animals transfected with the scrambled oligonucleotide. Arrows and arrowheads indicate double-labeled cells and cells that are GFP positive but marker negative, respectively. Scale bars, 50 μm. (C and E) Quantitation of sections similar to those in (B) and (D). Transfected cells within the SVZ (C) or CP (E) of four scrambled and four gp130 siRNA electroporated animals (four sections per brain) were counted. *$p < 0.05$, **$p < 0.01$ relative to scrambled oligonucleotide controls (ANOVA). Error bars indicate SEM. (F–H) Cardiotrophin-1 is expressed by developing cortical neurons. (F) RT-PCRs for CT-1 and LIF mRNAs in E12, E13, or E17.5 cortex and cultured cortical neurons (CN). GAPDH was used as a loading and genomic DNA contamination control. (G) Western blot for CT-1 in equal volumes of concentrated CM from *CT-1*$^{-/-}$ (KO) and *CT-1*$^{+/+}$ (WT) precursors cultured 4 DIV, or from developing cortical neurons (CN). Purified murine CT-1 was analyzed directly (10 ng, 5 ng) or after concentration in control medium ([10ng]). Asterisks indicate a band enriched in neuron-derived CM, clearly detected in *CT-1*$^{+/+}$ CM after a longer exposure (data not shown) but absent from *CT-1*$^{-/-}$ CM. (H) Immunostaining of E18 coronal cortical sections for CT-1 (red) and the neuronal protein HuD (green). Scale bar, 100 μm.

cytokine coreceptor, gp130, in embryonic cortical precursor cells.

CT-1 Is Produced by Newly Born Cortical Neurons and Is Responsible for the Gliogenic Effects of CM

Our RT-PCR analysis (Figure 2D) demonstrated that developing cortical neurons express at least three gliogenic cytokines, CNTF, LIF, and CT-1. Since CNTF is not expressed in the embryonic brain (Stockli et al., 1991), and since *CNTF*$^{-/-}$ mice have no alterations in astrocyte number in the adult brain (Barres et al., 1996; Martin et al., 2003), we focused upon CT-1, which is expressed in the embryonic cortex (Oppenheim et al., 2001) and LIF, which is expressed in neurons throughout the adult brain (Lemke et al., 1996). Initially, we characterized expression of CT-1 and LIF in vivo. RT-PCR analysis of the E12 to E17.5 cortex revealed that CT-1 was barely detectable at E12 or E13 but was readily detectable by E17.5 (Figure 6F), confirming previous analyses (Oppenheim et al., 2001; Sheng et al., 1996). In contrast, LIF mRNA was undetectable in the embryonic cortex (Figure 6F). To ask what cells expressed CT-1 in vivo, we performed immunocytochemistry on the E18 cortex and demonstrated that CT-1 was enriched in HuD- and NeuN-positive neurons in the cortical plate but was not detectably expressed in the VZ/SVZ (Figure 6H; Figure S3D). The specificity of this immunostaining was demonstrated by preabsorbing the antibody with CT-1 (Figure S3D). To further confirm that embryonic cortical neurons synthesize and secrete CT-1, we cultured neurons or cortical precursors from wild-type or *CT-1*$^{-/-}$ mice (Oppenheim et al., 2001) for 4 days and generated CM from these cultures for 1 additional day. Western blots of the concentrated CM demonstrated that a CT-1 immunoreactive band was detectable in CM generated from *CT-1*$^{+/+}$ but not *CT-1*$^{-/-}$ precursors and that this same immunoreactive protein was present at higher levels in CM from cortical neurons (Figure 6G).

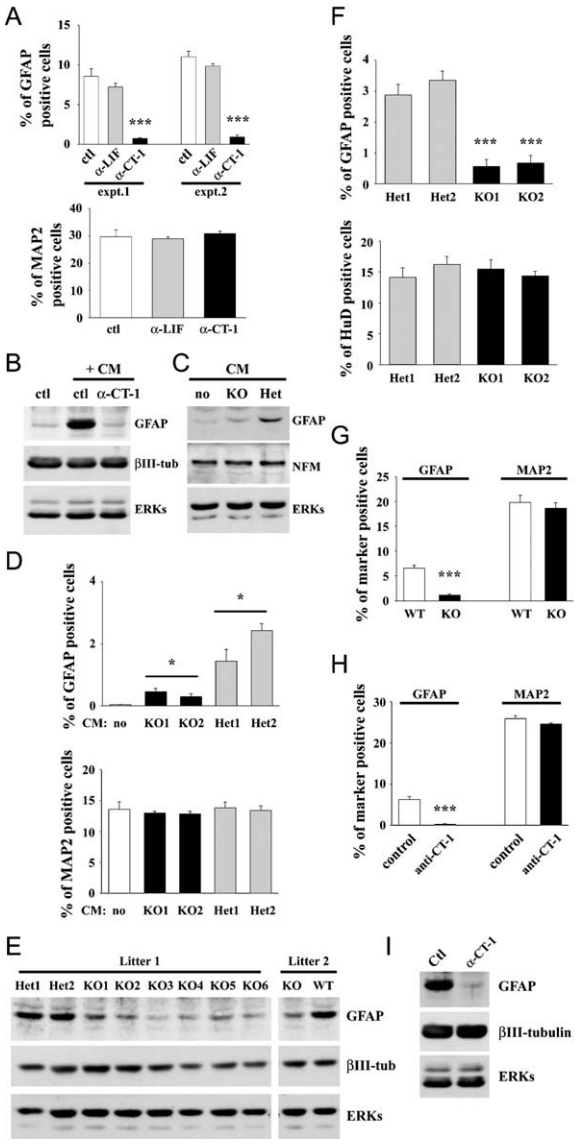

Figure 7. Cardiotrophin-1 but Not LIF Is Essential for Appropriate Astrogliogenesis in Culture

(A) Percentage of GFAP- and MAP2-positive cells in precursors cultured 5 days in 4 DIV CM with or without control goat IgG, CT-1 function-blocking antibody (expt. 1 and expt. 2), or LIF function-blocking antibody (expt. 2). Alternatively, CM was depleted for LIF by immunoprecipitation with a different LIF antibody prior to addition to cells (expt. 1). ***$p < 0.001$, ANOVA. Error bars indicate SEM.

(B) Western blots of precursors grown 5 days in control medium, or in 4 DIV CM plus CT-1 function-blocking antibody or control goat IgG and probed for GFAP, βIII-tubulin, and total ERKs.

(C and D) Wild-type precursors cultured 4 days with control medium or with 4 DIV CM from *CT-1*$^{-/-}$ (KO) or *CT-1*$^{+/-}$ (Het) precursors. (C) Western blots for GFAP, NFM, and total ERKs. (D) Percentage of cells immunoreactive for GFAP or MAP2 in these cultures. *$p < 0.05$, KO versus no CM and Het versus KO, ANOVA. Representative results from one of three experiments are shown in each of (B)–(D). Error bars indicate SEM.

(E–G) Precursors were isolated from *CT-1*$^{-/-}$ (KO), *CT-1*$^{+/-}$ (Het), or *CT-1*$^{+/+}$ (WT) embryos, cultured for 6 days, and analyzed by Western blots for GFAP, βIII-tubulin, and total ERKs as a loading control (E) or immunocytochemically for astrocytic and neuronal markers (F and G). (F and G) Percentage of cells immunoreactive for GFAP (F and G), HuD (F), or MAP2 (G) in *CT-1*$^{-/-}$ versus *CT-1*$^{+/-}$ cultures. (F) and (G) derive from independent experiments. ***$p < 0.001$, ANOVA (F) and Student's t test (G). Error bars indicate SEM.

(H and I) Precursors were grown for 6 days in control medium supplemented with either goat IgG (control) or anti-CT-1 function-blocking antibody (anti-CT-1). (H) Quantitation of the number of cells immunoreactive for GFAP or MAP2. ***$p < 0.001$, Student's t test. Representative results from one of three experiments. Error bars indicate SEM. (I) Western blots for GFAP, βIII-tubulin, and total ERKs.

To ask whether CT-1 or LIF was responsible for the gliogenic effect of CM, E13 precursor cell cultures were incubated with 4 DIV CM in the presence or absence of function-blocking antibodies to either CT-1 (Chen and von Bartheld, 2004) or LIF (Mi et al., 2001). Immunocytochemistry and Western blots for GFAP and CD44 (Figures 7A and 7B and data not shown) 4 days later demonstrated that anti-CT-1 almost completely blocked the gliogenic effects of CM, while anti-LIF had no effect. In contrast, neither antibody affected the numbers of MAP2- or βIII-tubulin-positive neurons (Figure 7A). As further confirmation, we generated 4 DIV CM from *CT-1*$^{-/-}$ versus *CT-1*$^{+/-}$ cortical precursors and treated wild-type cultures with these different CM. Immunocytochemistry and Western blots 4 days later revealed that the number of astrocytes, but not neurons, was significantly reduced in cultures treated with *CT-1*$^{-/-}$ versus *CT-1*$^{+/-}$ CM (Figures 7C and 7D). Thus, CT-1 is produced by developing cortical neurons and is a major CM gliogenic factor.

Neuron-Derived CT-1 Is Essential for Normal Gliogenesis in Culture and In Vivo

These data suggest that neuron-derived CT-1 provides a feedback mechanism essential for the genesis of astrocytes. One prediction of this model is that loss of CT-1 should lead to a delay and/or reduction in endogenous astrocyte formation. To test this prediction, we cultured cortical precursors from *CT-1*$^{-/-}$ versus *CT-1*$^{+/-}$ or *CT-1*$^{+/+}$ littermates and analyzed them at 6 DIV. Immunocytochemical and Western blot analyses revealed a dramatic reduction in the numbers of GFAP-positive astrocytes in these cultures, with no significant effect on the numbers of neurons (Figures 7E–7G). Confirmation of this result was obtained by analyzing wild-type precursors cultured with function-blocking CT-1 antibody. Immunocytochemistry and Western blots demonstrated that anti-CT-1 blocked the endogenous genesis of cells expressing both GFAP and CD44 in these cultures (Figures 7H and 7I; data not shown) without affecting neurogenesis. Thus, endogenous CT-1 is essential for endogenous astrogenesis in culture.

We then asked whether CT-1 was also important in vivo by analyzing the cortex of neonatal *CT-1*$^{-/-}$ mice, utilizing both GFAP and CD44 as astrocytic markers. To confirm that CD44 was expressed in early cortical astrocytes in vivo as we had demonstrated in vitro (Figures S1B and S1C), we performed double-label immunocytochemistry for CD44 and GFAP. At P1, the large majority of CD44-positive cells were present in the VZ/SVZ, and almost all of them were GFAP positive (Figure 8A). Conversely, many of the GFAP-positive cells in the SVZ were CD44 positive. We then performed Western blot analysis for CD44 and GFAP in the P0 or P1 *CT-1*$^{-/-}$ versus *CT-1*$^{+/+}$ cortex. This analysis revealed reduced levels of both of these astrocyte

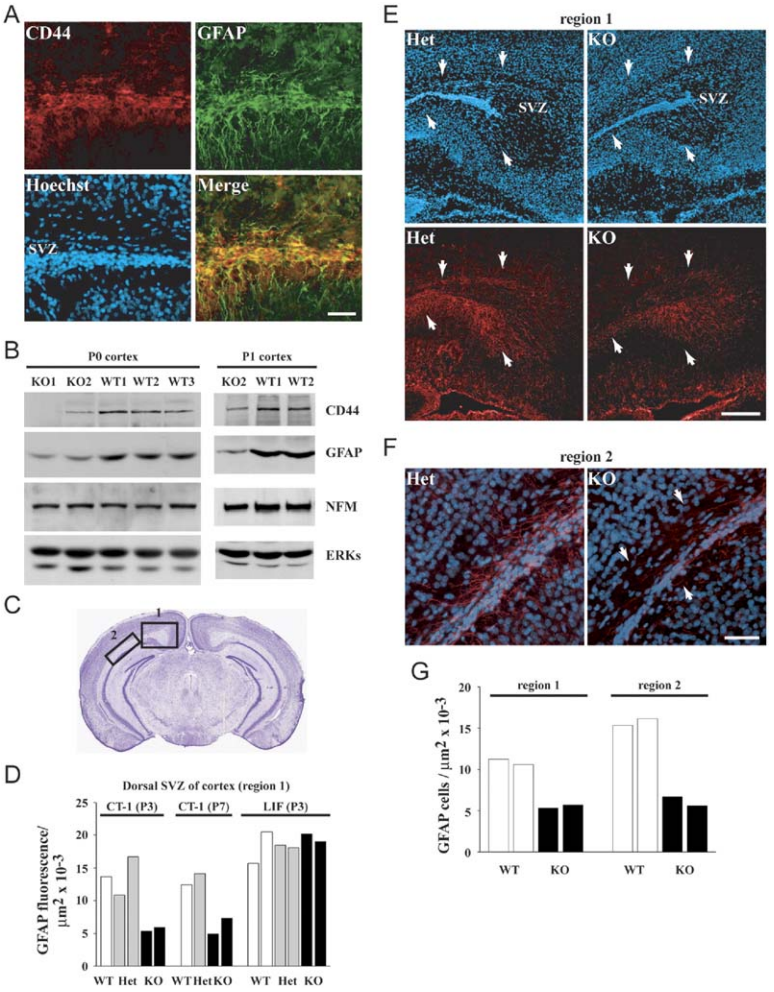

Figure 8. Cardiotrophin-1 but Not LIF Is Essential for Appropriate Cortical Astrogenesis In Vivo

(A) Photomicrographs of a coronal section through the neonatal cortical SVZ double-labeled for CD44 (red) and GFAP (green) and counterstained with Hoechst 33258 (blue). The merge shows just the CD44 and GFAP fluorescence. Scale bar, 50 μm.

(B) Cortices isolated from P0 (left) or P1 (right) *CT-1*$^{-/-}$ (KO) or *CT-1*$^{+/+}$ (WT) mice and analyzed by Western blots for CD44, GFAP, NFM, or total ERKs.

(C–F) P3 or P7 CT-1 or P3 *LIF*$^{-/-}$ (KO), +/- (Het), and +/+ (WT) brains were analyzed immunocytochemically for GFAP. (C) Schematic of the quantified regions of the caudal cortical SVZ (boxed areas). (D) GFAP fluorescence intensity per defined area from sections similar to those in (E) through SVZ region 1 as defined in (C). (E and F) GFAP immunofluorescence (red) in SVZ regions 1 (E) and 2 (F) of P3 *CT-1*$^{+/-}$ (Het) versus *CT-1*$^{-/-}$ (KO) mice. SVZ regions were defined by Hoechst labeling (blue). Arrows highlight regions of reduced GFAP staining in the knockouts. Scale bars, 100 (E) or 50 (F) μm. (G) GFAP-positive cell density in P3 *CT-1*$^{-/-}$ (KO) or *CT-1*$^{+/+}$ (WT) cortical regions 1 and 2 as analyzed by DAB immunocytochemistry.

proteins in the absence of CT-1, while no differences were observed in levels of NFM (Figure 8B). To further characterize this reduction, we performed GFAP immunocytochemistry on the *CT-1*$^{-/-}$ cortex at P3 and P7 and quantitated the immunofluorescence signal in a defined region of the dorsal SVZ (region 1; Figures 8C–8F). This analysis demonstrated a 50%–75% reduction in GFAP signal throughout the *CT-1*$^{-/-}$ SVZ at both of these time points (Figure 8D), in good agreement with the Western blot data (Figure 8B). In contrast, a similar analysis of the dorsal SVZ of P3 *LIF*$^{-/-}$ animals showed no reduction in GFAP fluorescence intensity (Figure 8D). Finally, to confirm that this decrease in astrocytic proteins reflected a decrease in astrocyte numbers, we quantitated the density of GFAP-positive cells in two defined regions of the cortical SVZ (Figure 8C) using DAB immunohistochemistry. This analysis revealed that the density of GFAP-positive astrocytes in the P3 *CT-1*$^{-/-}$ cortex was decreased 50%–75% relative to the *CT-1*$^{+/+}$ cortex (Figure 8G). Thus, neuron-derived CT-1 but not LIF is essential in vivo for appropriate gliogenesis in the developing cortex.

Discussion

These results support three major conclusions. First, our culture studies indicate that newly born cortical neurons express gliogenic cytokines that instruct multipotent cortical precursors to generate astrocytes, thereby regulating the temporal onset of gliogenesis. Second, our in vivo work indicates that early cortical precursors are competent to produce astrocytes but fail to do so because they require gliogenic cytokine signaling that only occurs late during embryogenesis. Third, we demonstrate that the cytokine CT-1 is highly enriched in embryonic cortical neurons, and this neuron-derived CT-1 is essential for astrogenesis in culture and in vivo. Together, these findings support a developmental mechanism by which cortical precursors first generate neurons that produce and secrete the cytokine CT-1, which then provides a feedback loop to direct the parent cortical precursors to start making astrocytes. Such a mechanism provides an unanticipated level of crosstalk between these different developing cellular populations and ensures that astrocytes are made only after sufficient numbers of neurons are generated.

Our in utero electroporation data argue that cortical precursors are competent to generate astrocytes in response to cytokines as early as E13/E14 in vivo and from E11.5 in vitro. That they do not do so argues that environmental cytokine levels are limiting, in spite of the recent finding that two cytokines, neuropoietin and cardiotrophin-like cytokine, are expressed in embryonic mouse neuroepithelia (Uemura et al., 2002;

Derouet et al., 2004). Consistent with this conclusion, Morrow et al. (2001) showed that the postnatal but not embryonic cortical environment directed embryonic cortical precursors to generate astrocytes. Our data indicate that this difference in cortical environment is at least partially due to neuron-derived CT-1. These findings do not, however, exclude the possibility that prior to E13/E14, cortical precursors undergo an intrinsic change such as demethylation of STAT binding sites on astrocyte gene promoters (Takizawa et al., 2001) that allows them to respond to cytokines. Moreover, other intrinsic alterations such as EGFR expression (Burrows et al., 1997) or interactions between the JAK-STAT pathway and HLH proteins and/or the Notch pathway (Kamakura et al., 2004; He et al., 2005) may render cortical precursors more responsive to low cytokine levels. Nonetheless, our data strongly argue that neuron-derived CT-1 provides a key extrinsic signal for determining when and how many astrocytes are generated in the developing cortex.

The idea that cytokines are important for astrogliogenesis is well supported; CNTF, LIF, and CT-1 all promote gliogenesis in cultured precursors via a gp130-JAK-STAT pathway (Bonni et al., 1997; Rajan and McKay, 1998; Nakashima et al., 1999b; Ochiai et al., 2001), and while mice lacking CNTF and LIF do not display deficits in adult astrocyte number (Barres et al., 1996; Martin et al., 2003) or cortical gliogenesis (data reported here), respectively, mice lacking gp130 or LIFR have significantly reduced numbers of CNS astrocytes (Ware et al., 1995; Koblar et al., 1998; Nakashima et al., 1999a). Here, we demonstrate that neuron-derived CT-1 is a key player in a feedback mechanism regulating the switch from neurogenesis to gliogenesis in the cortex. Since a similar developmental switch occurs throughout the developing CNS, and since deficits in astrocyte number are widespread in the *gp130*$^{-/-}$ and *LIFR*$^{-/-}$ mice, then such a neuron-based feedback mechanism may be important throughout the embryonic brain and spinal cord. Moreover, if neurons produce cytokines like CT-1 into adulthood, then this might explain why precursors transplanted into the postnatal brain or an injured CNS environment generate virtually exclusively glial cells (Morrow et al., 2001; Vroemen et al., 2003).

The deficit in astrocyte formation shown here is very dramatic in precursor cultures depleted of CT-1, and astrocyte numbers are reduced 50%–75% in the neonatal *CT-1*$^{-/-}$ cortex. However, while this is a robust decrease, it is not as dramatic as that seen in the *LIFR*$^{-/-}$ or *gp130*$^{-/-}$ mice (Ware et al., 1995; Koblar et al., 1998; Nakashima et al., 1999a). This difference may be due to the synthesis of other cytokines by developing neurons, as we demonstrate here. In this regard, since in vivo expression of LIF (shown here) and CNTF (Stockli et al., 1991) commences postnatally, and since adult *LIF*$^{-/-}$ mice have reduced numbers of hippocampal astrocytes (Koblar et al., 1998; Bugga et al., 1998), then we propose that these other cytokines provide compensatory/additional neuron-derived cytokines, particularly in a *CT-1*$^{-/-}$ background. Moreover, astrocytes (Lillien et al., 1988) can produce gliogenic cytokines like CNTF, suggesting that once the first astrocytes are made, they may themselves participate in determining astrocytic density. In this regard, potential compensatory effects are not likely due to upregulation of LIF or CNTF, since neither mRNA is increased in adult *CT-1*$^{-/-}$ mice (Oppenheim et al., 2001). However, previous work with facial motor neurons (Sendtner et al., 1996) demonstrated that compensation/collaboration can nonetheless occur; while *LIF*$^{-/-}$ mice showed no motor neuron degeneration, *LIF*$^{-/-}$, *CNTF*$^{-/-}$ mice displayed considerably enhanced neuronal degeneration relative to mice lacking CNTF alone, even though there was no upregulation of LIF mRNA in the *CNTF*$^{-/-}$ mice.

One surprising result reported here is that cytokines signal via the gp130-JAK-STAT pathway to inhibit neurogenesis. Interestingly, signals that promote cortical neurogenesis such as the MEK-C/EBP pathway (Ménard et al., 2002; Barnabé-Heider and Miller, 2003; A. Paquin, F.B.-H., R. Kageyama, and F.D.M., unpublished data) and neurogenic bHLHs (Sun et al., 2001), also inhibit gliogenesis. Thus, in both the CNS and PNS (Shah et al., 1994), growth factors in the embryonic neural environment actively bias precursors toward one fate and against another.

The idea of developmental timers is well established, but in most cases the underlying mechanism is thought to be a cell-intrinsic clock that is tied to the cell cycle (reviewed in Raff et al., 2001; Bessho and Kageyama, 2003). Here, we present a different timer mechanism, by which one class of daughter cells, neurons, secretes CT-1, thereby altering the environment of the parent precursors to favor production of a second class of daughter cells, astrocytes. Thus, these findings not only provide insights into cortical development, but also raise the possibility that a similar feedback mechanism may underlie the sequential genesis of cell types in other developing systems.

Experimental Procedures

Cortical Precursor and FACS-Purified Neuron Cultures

Cortical precursors and postmitotic neurons were cultured as described in 40 ng/ml FGF2 (Ménard et al., 2002; Barnabé-Heider and Miller, 2003) from CD1 mice, actin:EYFP transgenic mice (Hadjantonakis et al., 1998), K6 Tα1:nlacZ transgenic mice (Gloster et al., 1999), or *CT-1*$^{-/-}$ mice (Oppenheim et al., 2001) at a density of 200,000 cells/ml with, for clonal cultures, 1500 cells/ml of transgenic cells. For sorted neurons, cortical tissue from aged-matched K6 or control CD1 embryos were dissociated with papain (Schinelli et al., 1994), labeled with propidium iodide (20 ng/ml; Sigma) and FDG (Molecular Probes), sorted using Dako Cytomation MoFlo, and cultured as above. CM was collected by filtration through 0.22 μm pores and supplemented with B27, glutamine, and FGF2 for addition to precursor cells at 90% (50% for heat-denatured medium). CNTF, CT-1 (both Cedarlane Laboratories), and LIF (Sigma) were used at 50 ng/ml.

Inhibitor Treatments and Transfection of Precursor Cultures

AG490 5 μM (Calbiochem) and PD98059 50 μM (BIOMOL Research Laboratories) in 1% DMSO were added after 3 DIV, and cultures were analyzed 2 days later. CT-1 or LIF function-blocking antibodies or control goat IgG was added at the time of plating (5 μg/ml; R&D Systems). CM was depleted of LIF by incubation overnight at 4°C with a rabbit LIF antibody (5 μg/ml, Santa Cruz), addition of 30 μl of protein A Sepharose beads (Sigma) for 1 hr, and centrifugation. For transfections, plasmids used were pEGFP (Clonetech), pSilencer (Ambion) with or without a STAT3 siRNA sequence (5′-TCAGGTTGCTGGTCAAATT-3′), or the pCAGGS expression vector with or without a rat CNTF sequence engineered for secretion (the kind gift of Dr. Miyazaki [Jiang et al., 2003]). Three hours after plating, precursors were transfected using Fugene6 (Roche), mixing

0.33 μg pEGFP, 0.66 μg STAT3 siRNA or CNTF plasmids, 1.5 μl Fugene6, and 100 μl OptiMEM (Invitrogen). Alternatively, precursors were transfected at 1 DIV with 60 pmol of siRNA oligonucleotides targeted to gp130 (proprietary mixture of four siRNAs from Dharmacon) or a nonspecific scrambled sequence (5′-AAAGGTTAGCCTA CATTACAG-3′) with 2 μg EGFP using Lipofectamine 2000 (Invitrogen). Cos cells and NIH-3T3 cells were transfected using Lipofectamine 2000.

In Utero Electroporation

In utero electroporation was performed as described (Ohtsuka et al., 1999) with E13.5– E15 CD1 mice by injecting a nuclear GFP expression plasmid driven from the EF1 promoter (the kind gift of Dr. R. Kageyama) (5 μg/μl) with 20 pmol siRNA or a 1:3 ratio of GFP:pCAGGS (5 μg/μl) and 0.05% trypan blue as a tracer. The square electroporator CUY21 EDIT (TR Tech, Japan) was used to deliver five 50 ms pulses of 50 V with 950 ms intervals per embryo. Brains were fixed in 4% PFA at 4ºC overnight, cryoprotected, and cryosectioned coronally at 16 μm.

Immunocytochemistry and Quantitation

Immunocytochemistry on cells was performed as described (Toma et al., 2000; Barnabé-Heider and Miller, 2003). For immunostaining of tissue sections, sections were postfixed with 4% PFA, blocked, and permeabilized with 10% BSA and 0.3% Triton-X, and then with the M.O.M. blocking kit (Vector Laboratories). Sections were incubated with primary antibodies at 4ºC overnight, with secondary antibodies at room temperature for 1 hr, counterstained with Hoechst 33258 (1:2000; Sigma-Aldrich) and mounted with GelTol (Fisher). All antibodies are listed in the Supplemental Data. For quantitation, six to eight random fields of cultured cells (per treatment per experiment) were captured and processed. For quantitation of GFAP immunohistochemistry from neonatal *CT-1*$^{-/-}$ and *LIF*$^{-/-}$ mice (Oppenheim et al., 2001; Sendtner et al., 1996), tissue sections were selected from identical rostrocaudal levels of −/−, +/−, and +/+ littermates. Cell counts were made from defined regions on sections processed using DAB. For unbiased GFAP fluorescence measurements, Hoechst-labeled fields were used to define regions of the SVZ, and fluorescence was automatically captured from the corresponding GFAP image. Digital image acquisition and analysis were performed using Northern Eclipse software (Empix Inc.) with a Sony XC-75CE CCD video camera. For electroporated brains, sections were analyzed using a Zeiss Pascal confocal microscope and the manufacturer's software. Two to three pictures per coronal section were taken with a 20× objective, and a total of four sections per animal were analyzed. In all graphs, error bars indicate standard error of the mean (SEM), and statistics were performed using one-way ANOVA with the Student-Newman-Keuls post hoc test or Student's t test, as appropriate.

Western Blot Analysis and Biochemistry

Cortical precursors and neonatal cortices were lysed, and Western blots were performed as described (Barnabé-Heider and Miller, 2003). For acute stimulation experiments, precursors were first washed for 1 hr. For CT-1 analysis, primary cells were cultured for 4 days and switched to OptiMEM for 24 hr, and the resultant CM was concentrated using iCON concentrator filters (9 kDa cut-off; Pierce) and analyzed by SDS-PAGE. Membranes were blocked in 5% skim milk powder, or in 3% BSA for phospho-JAK2, CD44, and CT-1 antibodies. All antibodies are listed in the Supplemental Data.

RT-PCR Analysis

RNA was extracted using Trizol reagent (Invitrogen) and treated with DNase (Fermentas) for 30 min at 37ºC. cDNA was synthesized from 300–1000 ng of RNA using murine leukemia virus reverse transcriptase (Fermentas). After reverse transcription, 1 μl of each RT mixture was subjected to PCR using Taq polymerase (Fermentas) under the following conditions: 95ºC for 9 min, 40 cycles of 94ºC 20 s, 60ºC 20 s, 72ºC 30 s, and 72ºC 5 min. Primers are listed in the Supplemental Data.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures and three supplemental figures and can be found with this article online at http://www.neuron.org/cgi/content/full/48/2/253/DC1/.

Acknowledgments

This work was supported by grants from the CIHR to F.D.M. and the NCIC to D.R.K. F.D.M. and D.R.K. are CRC Senior Research Chairs, F.B.-H. was supported by CIHR and McGill Tomlinson studentships, and J.A.W. has an NSERC fellowship. We thank Dr. Kageyama and members of his laboratory for teaching F.B.-H. the in utero electroporation technique; Dr. Miyazaki and Dr. Raptis for the gifts of the CNTF and STAT3 siRNA plasmids; Dr. Sherry Zhao for operating the FACS equipment; and Dr. Stefan Weiss for technical assistance with CT-1 and LIF knockout mice.

Received: August 18, 2004
Revised: May 17, 2005
Accepted: August 25, 2005
Published: October 19, 2005

References

Barnabé-Heider, F., and Miller, F.D. (2003). Endogenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathways. J. Neurosci. *23*, 5149–5160.

Barres, B.A., Burne, J.F., Holtmann, B., Thoenen, H., Sendtner, M., and Raff, M.C. (1996). CNTF enhances the rate of oligodendrocyte generation. Mol. Cell. Neurosci. *8*, 146–156.

Bessho, Y., and Kageyama, R. (2003). Oscillations, clocks and segmentation. Curr. Opin. Genet. Dev. *13*, 379–384.

Bonni, A., Sun, Y., Nadal-Vicens, M., Bhatt, A., Frank, D.A., Rozovsky, I., Stahl, N., Yancopoulos, G.D., and Greenberg, M.E. (1997). Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. Science *278*, 477–483.

Bugga, L., Gadient, R.A., Kwan, K., Stewart, C.L., and Patterson, P.H. (1998). Analysis of neuronal and glial phenotypes in brains of mice deficient in leukemia inhibitory factor. J. Neurobiol. *36*, 509–524.

Burrows, R.C., Wancio, D., Levitt, P., and Lillien, L. (1997). Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex. Neuron *19*, 251–267.

Calegari, F., Haubensak, W., Yang, D., Huttner, W.B., and Buchholz, F. (2002). Tissue specific RNA interference in postimplantation mouse embryos with endoribonuclease prepared short interfering RNA. Proc. Natl. Acad. Sci. USA *99*, 14236–14240.

Chen, J., and von Bartheld, C.S. (2004). Role of exogenous and endogenous trophic factors in the regulation of extraocular muscle strength during development. Invest. Ophthalmol. Vis. Sci. *45*, 3538–3545.

Derouet, D., Rousseau, F., Alfonsi, F., Froger, J., Hermann, J., Barbier, F., Perret, D., Diveu, C., Guillet, C., Preisser, L., et al. (2004). Neuropoietin, a new IL-6-related cytokine signaling through the ciliary neurotrophic factor receptor. Proc. Natl. Acad. Sci. USA *101*, 4827–4832.

Gloster, A., El-Bizri, H., Bamji, S.X., Rogers, D., and Miller, F.D. (1999). Early induction of Tα1 α-tubulin transcription in neurons of the developing nervous system. J. Comp. Neurol. *405*, 45–60.

Hadjantonakis, A.K., Gertsenstein, M., Ikawa, M., Okabe, M., and Nagy, A. (1998). Generating green fluorescent mice by germline transmission of green fluorescent ES cells. Mech. Dev. *76*, 79–90.

He, F., Ge, W., Martinowich, K., Becker-Catania, S., Coskun, V., Zhu, W., Wu, H., Castro, D., Guillemot, F., Fan, G., et al. (2005). A positive autoregulatory loop of Jak-STAT signaling controls the onset of astrogliogenesis. Nat. Neurosci. *8*, 616–625.

Jiang, J., Yamato, E., and Miyazaki, J. (2003). Long-term control of food intake and body weight by hydrodynamics-based delivery of plasmid DNA encoding leptin or CNTF. J. Gene Med. *5*, 977–983.

Kamakura, S., Oishi, K., Yoshimatsu, T., Nakafuku, M., Masuyama, N., and Gotoh, Y. (2004). Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signaling. Nat. Cell Biol. *6*, 547–554.

Koblar, S.A., Turnley, A.M., Classon, B.J., Reid, K.L., Ware, C.B., Cheema, S.S., Murphy, M., and Bartlett, P.F. (1998). Neural precursor differentiation into astrocytes requires signaling through the leukemia inhibitory factor receptor. Proc. Natl. Acad. Sci. USA *95*, 3178–3181.

Lemke, R., Gradient, R.A., Schliebs, R., Bigl, V., and Patterson, P.H. (1996). Neuronal expression of LIF in the rat brain. Neurosci. Lett. *215*, 205–208.

Lillien, L.E., Sendtner, M., Rohrer, H., Hughes, S.M., and Raff, M.C. (1988). Type-2 astrocyte development in rat brain cultures is initiated by a CNTF-like protein produced by type-1 astrocytes. Neuron *1*, 485–494.

Liu, Y., Han, S.S., Wu, Y., Tuohy, T.M., Xue, H., Cai, J., Back, S.A., Sherman, L.S., Fischer, I., and Rao, M.S. (2004). CD44 expression identifies astrocyte-restricted precursor cells. Dev. Biol. *276*, 31–46.

Martin, A., Hofmann, H.-D., and Kirsch, M. (2003). Glial reactivity in CNTF-deficient mice after optic nerve lesion. J. Neurosci. *23*, 5416–5424.

Ménard, C., Hein, P., Paquin, A., Savelson, A., Yang, X.M., Lederfein, D., Barnabé-Heider, F., Mir, A.A., Sterneck, E., Peterson, A.C., et al. (2002). An essential role for a MEK-C/EBP pathway during growth factor-regulated cortical neurogenesis. Neuron *36*, 597–610.

Mi, H., Haeberle, H., and Barres, B.A. (2001). Induction of astrocyte differentiation by endothelial cells. J. Neurosci. *21*, 1538–1547.

Morrow, T., Song, M.R., and Ghosh, A. (2001). Sequential specification of neurons and glia by developmentally regulated extracellular factors. Development *128*, 3585–3594.

Nakashima, K., Wiese, S., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Yoshida, K., Kishimoto, T., Sendtner, M., and Taga, T. (1999a). Developmental requirement of gp130 signaling in neuronal survival and astrocyte differentiation. J. Neurosci. *19*, 5429–5434.

Nakashima, K., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Kawabata, M., Miyazono, K., and Taga, T. (1999b). Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. Science *284*, 479–482.

Nieto, M., Schuurmans, C., Britz, O., and Guillemot, F. (2001). Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. Neuron *29*, 401–413.

Ochiai, W., Yanagisawa, M., Takizawa, T., Nakashima, K., and Taga, T. (2001). Astrocyte differentiation of fetal neuroepithelial cells involving cardiotrophin-1-induced activation of STAT3. Cytokine *14*, 264–271.

Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F., and Kageyama, R. (1999). Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. EMBO J. *18*, 2196–2207.

Oppenheim, R.W., Wiese, S., Prevette, D., Armanini, M., Wang, S., Houenou, L.J., Holtmann, B., Gotz, R., Pennica, D., and Sendtner, M. (2001). Cardiotrophin-1, a muscle derived cytokine, is required for the survival of subpopulations of developing motoneurons. J. Neurosci. *21*, 1283–1291.

Qian, X., Shen, Q., Goderie, S.K., He, W., Capela, A., Davis, A.A., and Temple, S. (2000). Timing of CNS cell generation: A programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. Neuron *28*, 69–80.

Raff, M., Apperly, J., Kondo, T., Tokumoto, Y., and Tang, D. (2001). Timing cell-cycle exit and differentiation in oligodendrocyte development. Novartis Found. Symp. *237*, 100–107; discussion 107–112, 158–163.

Rajan, P., and McKay, R.D. (1998). Multiple routes to astrocytic differentiation in the CNS. J. Neurosci. *18*, 3620–3629.

Sauvageot, C.M., and Stiles, C.D. (2002). Molecular mechanisms controlling cortical gliogenesis. Curr. Opin. Neurobiol. *12*, 244–249.

Schinelli, S., Paolillo, M., and Corona, G.L. (1994). Modulation of dopamine-induced cAMP production in rat striatal cultures by the calcium ionophore A23187 and by phorbol-12-myristate-13-acetate. Brain Res. Mol. Brain Res. *21*, 162–166.

Sendtner, M., Gotz, R., Holtmann, B., Escary, J.L., Masu, Y., Carroll, P., Wolf, E., Brem, G., Brulet, P., and Thoenen, H. (1996). Cryptic physiological trophic support of motoneurons by LIF revealed by double gene targeting of CNTF and LIF. Curr. Biol. *6*, 686–694.

Shah, N.M., Marchionni, M.A., Isaacs, I., Stroobant, P., and Anderson, D.J. (1994). Glial growth factor restricts mammalian neural crest stem cells to a glial fate. Cell *77*, 349–360.

Sheng, Z., Pennica, D., Wood, W.I., and Chien, K.R. (1996). Cardiotrophin-1 displays early expression in the murine heart tube and promotes cardiac myocyte survival. Development *122*, 419–428.

Stockli, K.A., Lillien, L.E., Naher-Moe, M., Breitfeld, G., Hughes, R.A., Raff, M.C., Thoenen, H., and Sendtner, M. (1991). Regional distribution, developmental changes, and cellular localization of CNTF-mRNA and protein in the rat brain. J. Cell Biol. *115*, 447–459.

Sun, Y., Nadal-Vicens, M., Misono, S., Lin, M.Z., Zubiaga, A., Hua, X., Fan, G., and Greenberg, M.E. (2001). Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. Cell *104*, 365–376.

Sun, Y., Goderie, S.K., and Temple, S. (2005). Asymmetric distribution of EGFR receptor during mitosis generates diverse CNS progenitor cells. Neuron *45*, 873–886.

Takizawa, T., Nakashima, K., Namihara, M., Ochiai, W., Uemura, A., Yanagisawa, M., Fujita, N., Nakao, M., and Taga, T. (2001). DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. Dev. Cell *1*, 749–758.

Toma, J.G., El-Bizri, H., Barnabé-Heider, F., Aloyz, R., and Miller, F.D. (2000). Evidence that helix-loop-helix proteins collaborate with retinoblastoma tumor suppressor protein to regulate cortical neurogenesis. J. Neurosci. *20*, 7648–7656.

Uemura, A., Takizawa, T., Ochiai, W., Yanagisawa, M., Naiashima, K., and Taga, T. (2002). Cardiotrophin-like cytokine induces astrocyte differentiation of fetal neuroepithelial cells via activation of STAT3. Cytokine *18*, 1–7.

Vroemen, M., Aigner, L., Winkler, J., and Weidner, N. (2003). Adult neural progenitor cell grafts survive after acute spinal cord injury and integrate along axonal pathways. Eur. J. Neurosci. *18*, 743–751.

Ware, C.B., Horowitz, M.C., Renshaw, B.R., Hunt, J.S., Liggitt, D., Koblar, S.A., Gliniak, B.C., McKenna, H.J., Papayannopoulou, T., Thoma, B., et al. (1995). Targeted disruption of the low-affinity leukemia inhibitory factor receptor gene causes placental, skeletal, neural and metabolic defects and results in perinatal death. Development *121*, 1283–1299.