

Sequential specification of neurons and glia by developmentally regulated extracellular factors

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Accepted 28 June 2001

SUMMARY

Cortical progenitor cells give rise to neurons during embryonic development and to glia after birth. While lineage studies indicate that multipotent progenitor cells are capable of generating both neurons and glia, the role of extracellular signals in regulating the sequential differentiation of these cells is poorly understood. To investigate how factors in the developing cortex might influence cell fate, we developed a cortical slice overlay assay in which cortical progenitor cells are cultured over cortical slices from different developmental stages. We find that embryonic cortical progenitors cultured over embryonic cortical slices differentiate into neurons and those cultured over postnatal cortical slices differentiate into glia, suggesting that the fate of embryonic progenitors can be influenced by developmentally regulated signals. In contrast, postnatal progenitor cells differentiate into glial

cells when cultured over either embryonic or postnatal cortical slices. Clonal analysis indicates that the postnatal cortex produces a diffusible factor that induces progenitor cells to adopt glial fates at the expense of neuronal fates. The effects of the postnatal cortical signals on glial cell differentiation are mimicked by FGF2 and CNTF, which induce glial fate specification and terminal glial differentiation respectively. These observations indicate that cell fate specification and terminal differentiation can be independently regulated and suggest that the sequential generation of neurons and glia in the cortex is regulated by a developmental increase in gliogenic signals.

Key words: Cerebral cortex, Progenitor cells, Cell specification, Cell signalling, Glia, Neuron, Mouse

INTRODUCTION

One of most important aspects of the cellular organization of the nervous system is the presence of a large number of differentiated cell types that can mediate distinct functions. During development, differentiated cell types are generated from dividing progenitor cells. The mechanisms by which the principal cell types of the nervous system, neurons and glia, are generated from the progenitor population has been an area of active investigation for several years, and these studies have provided a number of important insights. In most parts of the nervous system neurons and glia are generated in sequence from a progenitor cell population and at least a subset of these progenitors are capable of generating both cell types. Many of these multipotent progenitors can also produce daughter cells that retain the ability to give rise to both neurons and glia, and by analogy with the hematopoietic system these cells are referred to as neural stem cells (reviewed in Patterson, 1990; McConnell, 1991; Alvarez-Buylla and Lois, 1995; Temple and Qian, 1996; Morrison et al., 1997).

Stem cells can generate differentiated progeny such as neurons and glia by either cell autonomous or non-cell autonomous mechanisms. For example, a stem cell might undergo asymmetric division to give rise to a stem cell

daughter and a daughter cell with a restricted proliferative and differentiation capacity. Alternatively, a stem cell might give rise to uncommitted progeny that are capable of differentiating along several different pathways in response to extracellular signals. The extracellular signal might select for a particular cell type in one of two ways. The signal might act by instructing a multipotent cell to adopt a specific developmental fate (instruction), or it might act as a survival factor for a lineage-committed daughter cell (selection). The sequential generation of neurons and glia in the cerebral cortex offers a good model system for evaluating the instructive and selective effects of extracellular factors, as well as cell autonomous mechanisms, in cell fate specification.

In the rodent cerebral cortex, neurons are generated from embryonic day 12 (E12) through E20, and glia are generated postnatally (Bayer and Altman, 1991). Lineage studies indicate that the developing cortex contains multipotent progenitor cells capable of generating both neurons and glia (Kilpatrick et al., 1995; Luskin et al., 1988; Reid et al., 1995; Walsh and Cepko, 1988; Walsh and Cepko, 1992), although separate precursors for neurons and glia have also been described (Davis and Temple, 1994; Luskin et al., 1993; Luskin et al., 1988; Price and Thurlow, 1988; Williams and Price, 1995). There are several mechanisms that might

contribute to the sequential generation of neurons and glia during cortical development. One possibility is that a multipotent stem cell gives rise to neuronal restricted progenitors early in development by asymmetric division, and gives rise to glial restricted progenitors late in development. The switch in cell fate could be regulated by a cell autonomous mechanism, for example by loss of intrinsic factors partitioned unequally during mitosis over time. Alternatively, the fate of cells generated at a particular division could be instructively specified by signals present in the extracellular environment. The presence of a neuronal fate inducing signal early in development and a glial fate inducing signal later in development could then account for the observed temporal pattern of cellular differentiation.

To analyze the influence of extracellular signals in cell fate decisions in the cortex, we have developed a cortical slice overlay assay that allows one to define the spatial and temporal distribution of signals that regulate differentiation without having a prior knowledge of the identity or nature of the signal. By culturing cortical progenitor cells directly on top of cortical slices we can investigate whether the sequential generation of neurons and glia in the cortex is an intrinsic property of the progenitor cells or if their fate is regulated by local environmental signals. Also, by performing clonal analysis on cells growing underneath cortical slices separated by a porous membrane, we can determine the nature of the signal, and whether the signals act instructively to alter cell fates or to selectively promote the survival of lineage restricted precursors. Here we describe experiments that show that the fate of early cortical progenitors can be influenced by local extracellular signals. We also show that a developmentally regulated extracellular signal induces glial cell fates, and that late cortical progenitors are restricted to glial fates. Finally, we show that FGF2 and CNTF influence cell fate and glial differentiation respectively, suggesting that these signals may co-operatively regulate the transition from neurogenesis to gliogenesis *in vivo*.

MATERIALS AND METHODS

Slice overlay cultures

Embryonic cortical progenitor cells were isolated at E15 from timed pregnant transgenic mice expressing the enhanced green fluorescent protein (EGFP) under the control of the β -actin promoter (Okabe et al., 1997). EGFP transgenic mice were identified with the aid of a UV lamp, dissected in HBSS, and the cells dissociated using papain (Threadgill et al., 1997). For E15 GFP cells, the whole cortex was used; for P5 GFP cells, only the subventricular zone was used. Coronal sections (250 μ m) of rat cortex between E18 and P15 were made using a vibratome, and 4–6 slices were then placed on poly-l-lysine- and laminin-coated membrane inserts in 6-well plates using an air-interface protocol (Polleux et al., 1998; Polleux et al., 2000). In each experiment, approximately 10^5 dissociated GFP cells were plated per well. The medium used was 70% basal Eagle's medium, 25% HBSS, 20 mM glucose, 1 mM glutamine, 1 mM penicillin-streptomycin, 1 mM kynurenic acid, and 5% horse serum (slice culture medium). One day later the medium was changed to slice culture medium without horse serum. Cells were cultured on top of cortical slices for 5–10 days. In some cases, 50 ng/ml of recombinant fibroblast growth factor (FGF2; Amgen), ciliary neurotrophic factor (CNTF; Upstate Biotechnology), platelet derived growth factor (BB

isoform; PDGF BB; Upstate Biotechnology), or epidermal growth factor (EGF; Upstate Biotechnology) was added to the medium under slices, 3 hours prior to the addition of GFP cells.

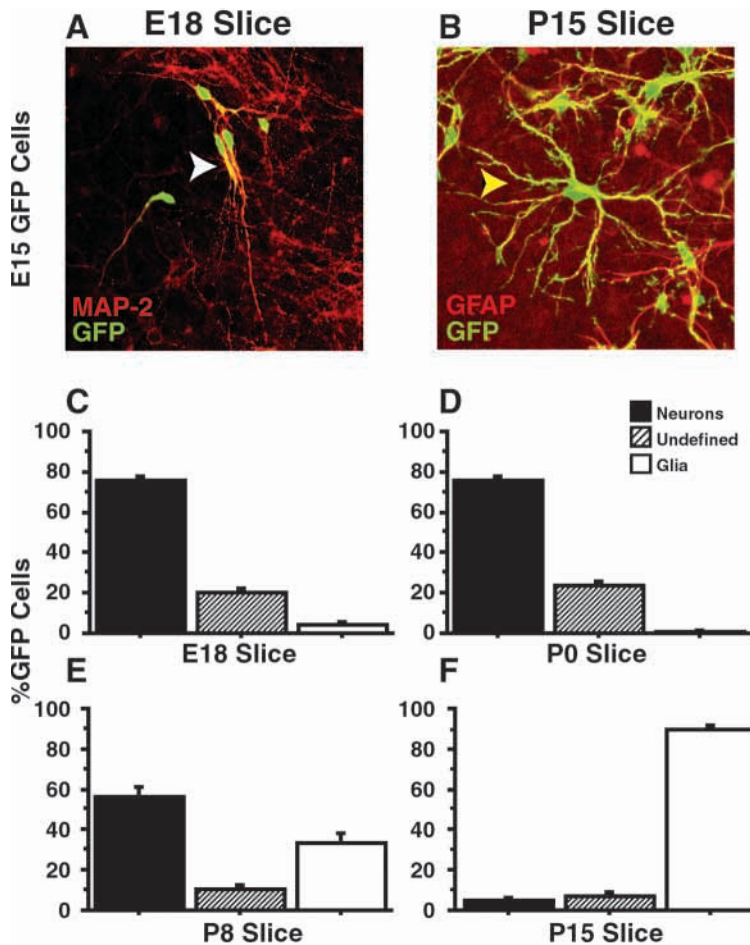
Immunostaining of slice overlay cultures on membranes and identification of cell types

Slices were fixed overnight with 4% paraformaldehyde (PFA) at 4°C and processed for immunofluorescence analysis by blocking nonspecific binding overnight at 4°C with 3% BSA, 0.3% Triton X-100, and 1% goat serum in PBS, followed by a further overnight 4°C incubation with primary antibody in 3% BSA and 0.3% Triton X-100 in PBS, and secondary antibodies in 3% BSA, 0.3% Triton X-100, and 1% goat serum in PBS. For BrdU immunofluorescence analysis slices were fixed overnight in 4% PFA, postfixed for 1 hour in 70% ethanol, permeabilized with 0.4% Triton X-100 in PBS for 1 hour, incubated with 2 N HCl for 30 minutes, 0.1 M NaB₂O₇ for 30 minutes, followed by overnight blocking, primary antibody incubation, and secondary antibody staining as above. The primary antibodies used were rabbit polyclonal anti-GFP (1:3000, Molecular Probes), mouse monoclonal anti-GFP (1:1000, Molecular Probes), mouse monoclonal anti-MAP-2 (1:3000, Sigma), mouse monoclonal anti-GFAP (1:1000, Sigma), mouse monoclonal anti-BrdU (1:400, Becton Dickinson) and rabbit polyclonal anti-NG2 (1:1000, Chemicon). The fluorescent secondary antibodies were goat anti-rabbit or goat anti-mouse Oregon Green-conjugated IgG (1:600, Molecular Probes), and goat anti-rabbit or goat anti-mouse Cy3-conjugated IgG (1:600, Molecular Probes). Cell nuclei were stained for 2 hours using Hoechst 33258 (1:2000, Molecular Probes). Images were acquired with a Nikon TE300 Eclipse microscope using IPLab Spectrum 3.2 (Scanalytics) or with a LSM 510 Zeiss confocal microscope equipped with helium-neon and argon lasers.

Most cultures at E15+5 days *in vitro* (DIV) were scored based on MAP-2 and GFP immunofluorescence since we did not find a definitive marker to identify astrocyte precursors (the RC2 antibody did not work well in our cultures), and mature astrocytic markers such as GFAP were not expressed at 5DIV. In these cultures, GFP-positive/MAP-2-positive (GFP+/MAP-2+) cells were scored as neurons. Neurons classified as pyramidal neurons had one major MAP-2-positive tapering process that frequently terminated in an apical tuft. These cells sometimes also had minor basilar dendrites, which were MAP-2 positive, but of finer caliber than the apical dendrite. GFP+/MAP-2-negative (MAP-2-) cells were scored as glial if they had a differentiated astrocytic morphology, and were scored as 'undefined' if they did not have a differentiated morphology. Cultures at E15+10DIV were scored as neurons or astrocytes based on GFP, MAP-2, and GFAP immunofluorescence.

Clonal analysis

Dissociated E15 cortical progenitor cells isolated from heterozygous GFP transgenic mice were diluted 1:1000 with wild-type progenitors from littermates and plated on poly-l-lysine- and laminin-coated glass coverslips in 12-well tissue culture plates (Costar) at 1×10^6 cells/well. Each well contained 1 ml of NEUROBASAL medium (Gibco) supplemented with glutamine (Gibco), penicillin-streptomycin (Gibco) and B27 (Gibco). GFP expression was used to confirm that the progenitors were completely dissociated and well isolated from each other so that clones could be unambiguously identified. To examine the influence of growth factors on clonal composition, cultures were treated with 10 ng/ml FGF2 (Amgen), 10 ng/ml PDGF BB (Upstate Biotechnology), 10 ng/ml ciliary neurotrophic factor (CNTF; Upstate Biotechnology), or 10 ng/ml EGF (Upstate Biotechnology) for the duration of the experiment. To determine if cortical slices produced diffusible signals that could influence clonal composition, cortical slices from E18 and P15 cortex were placed in membrane inserts, and the inserts were placed over glass coverslips in 12 well plates (Biocoat; Becton Dickinson) containing E15 GFP and E15 wild-type mixed cortical cultures as described above.



Immunostaining of dissociated cells on coverslips

Cells were fixed for 15 minutes at room temperature with 4% paraformaldehyde/4% sucrose, followed by blocking nonspecific binding for 2 hours with 3% BSA, 0.3% Triton X-100, and 1% goat serum in PBS. The cells were then incubated with primary antibody overnight at 4°C in with 3% BSA and 0.3% Triton X-100 in PBS, and then with secondary antibody at room temperature for 1 hour in 3% BSA, 0.3% Triton X-100 and 1% goat serum in PBS. The antibodies used were the same as described above.

Data analysis

All of the experiments involving slice overlay cultures were carried out at least three times, and analysis was based on quantification of at least 2 independent experiments with 15-20 slices scored per experimental condition. Approximately 200 cells were scored per slice. The experiments examining the effects of purified growth factors, and analysis of clone number over time were based on quantification of at least two independent experiments with at least 3 wells per experiment. Data are represented as mean \pm s.e.m.

RESULTS

Extracellular signals regulate cell fate decisions in the cerebral cortex

To determine if a developmentally regulated extracellular signal could influence the differentiation of embryonic cortical progenitor cells, we cultured E15 cells from GFP-expressing mice (GFP cells) (Okabe et al., 1997) on top of

Fig. 1. Extracellular signals in the cortex regulate neuronal and glial differentiation. (A,B) Dissociated E15 cortical GFP cells were cultured over rat E18 or P15 cortical slices for 5-10 days in vitro, and then processed for immunofluorescence to identify differentiated neurons and glia. Examples of E15 GFP cells growing over an E18 cortical slice after 5 days in vitro (A) or over P15 slice after 10 days in vitro (B). Cells growing over embryonic slices differentiated into MAP-2+ neurons with characteristic neuronal morphology (A), whereas cells growing on P15 slices differentiated into GFAP+ astrocytes with characteristic glial morphology (B). The white arrowhead in A identifies a neuron immunopositive for GFP (green channel) and the neuronal-specific dendritic marker MAP-2 (red channel; yellow on the merged image) while the yellow arrowhead in B identifies a glial cell immunopositive for GFP and the astrocyte specific marker GFAP. (C-F) Quantification of the number of GFP+ neurons (filled bars) and GFP+ glia (open bars) in slice overlay cultures from different developmental ages expressed as a percentage of the total number of GFP+ cells on top of the slice. The undefined population (hatched bars) were MAP-2- cells that failed to extend processes. Bar heights represent mean \pm s.e.m.

cortical slices taken from rats between embryonic day 18 (E18) and postnatal day 15 (P15). After 5-10 days in vitro, the differentiated phenotype of GFP cells was assessed by morphology as well as by double immunofluorescence using antibodies against GFP and the cell type-specific markers MAP-2 (neurons), GFAP (astrocytes), or NG-2 (oligodendrocytes) (Polleux et al., 1998; Polleux et al., 2000). After 5 DIV all of the cells with clearly identifiable axons and dendrites were MAP-2 positive and were classified as neurons (e.g. Fig. 1A). In these 5-day cultures the MAP-2-negative cells did not express mature glial markers, such as GFAP. These GFP+/MAP-2- cells were classified as glial if they had morphologies typical of immature astrocytes, or as 'undefined' if they were morphologically undifferentiated. By 10 DIV many of the GFP cells were immunopositive for GFAP (e.g. Fig. 1B), and therefore classification of cells in these cultures was based upon GFP, MAP-2 and GFAP immunofluorescence. The numbers of plated cells expressing oligodendrocyte markers was insignificant at all ages examined.

The phenotype of E15 GFP cells was strongly influenced by the age of the cortical slice on which the cells were plated (Fig. 1). When cells were plated over E18 slices, over 75% differentiated into pyramidal neurons with clearly identifiable axons and dendrites, and less than 10% differentiated into glia (Fig. 1A,C). In contrast, GFP cells plated over P15 postnatal slices differentiated almost exclusively into glial cells (>90%; Fig. 1B,F). There was a gradual developmental shift in the differentiated phenotype of GFP cells plated on slices from different ages. Whereas E18 and P0 slices supported similar levels of neuronal and glial differentiation (compare Fig. 1C and D), 55% of the E15 GFP cells plated on top of P8 slices differentiated into neurons and 30% differentiated into glia (Fig. 1E). Thus, the differentiation of embryonic cortical progenitor cells into neurons and glia was strongly influenced by the age of the slice over which the cells were cultured. Importantly, the glia-inducing activity was detected between

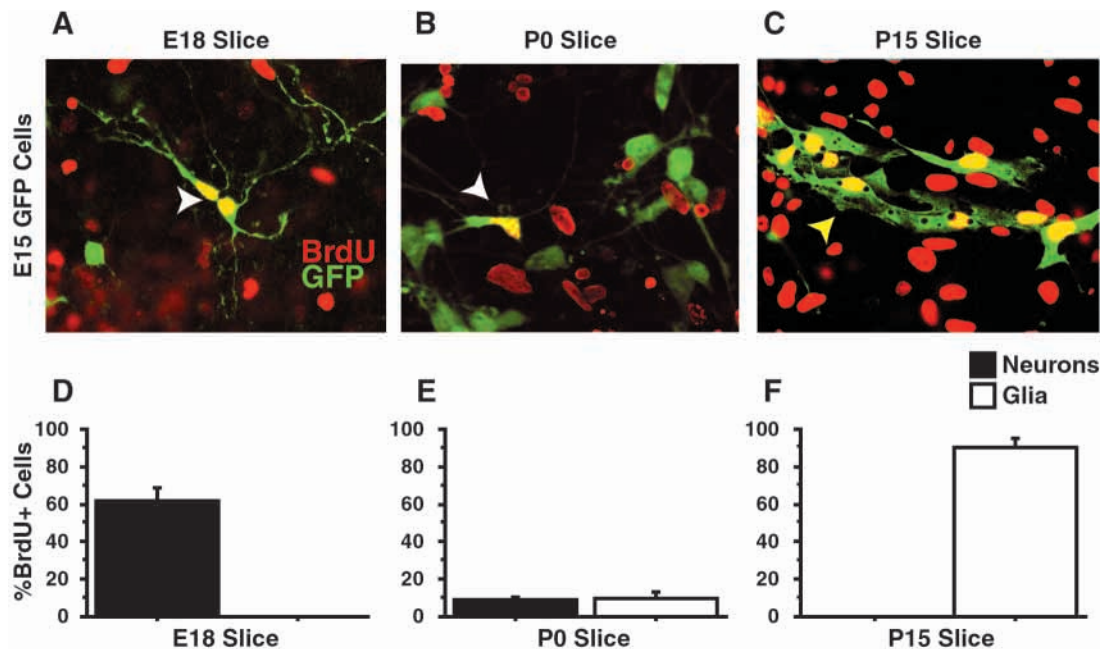


Fig. 2. A developmentally regulated signal can direct the fate of dividing embryonic cortical progenitor cells. Dissociated mouse E15 GFP cortical cells were cultured over rat E18, P0, or P15 cortical slices for 5 days *in vitro* in the presence of 10 μ M BrdU to identify progenitors that were proliferating at the time of plating. (A-C) Examples of E15 GFP cells on top of E18 slices (A), P0 slices (B), and P15 slices (C) that were processed for double immunofluorescence with antibodies directed against GFP (green channel) and BrdU (red channel). E15 GFP+/BrdU+ neurons are indicated by the white arrowheads (A and B) and GFP+/BrdU+ glia by the yellow arrowhead (C). (D-F) Quantification of the differentiated fate of dividing cortical progenitor cells plated on top of E18, P0, and P15 cortical slices. Neurons, filled bars; glia, open bars. Bar heights represent mean \pm s.e.m.

P0 and P8, which is at the onset of the gliogenic period in cortical development.

While these experiments indicated that factors present within the embryonic and postnatal cortical environment could influence the differentiation of cortical cells, they did not reveal whether these signals act upon dividing progenitor cells or upon newly postmitotic cells. To specifically examine the influence of the cortical environment on dividing progenitor cells, we labeled dividing cells by adding Bromodeoxyuridine (BrdU) to the medium, and subsequently examined the differentiated fate of dividing progenitor cells by double immunofluorescence for BrdU and GFP (in these experiments BrdU was present throughout the culture period). While a vast

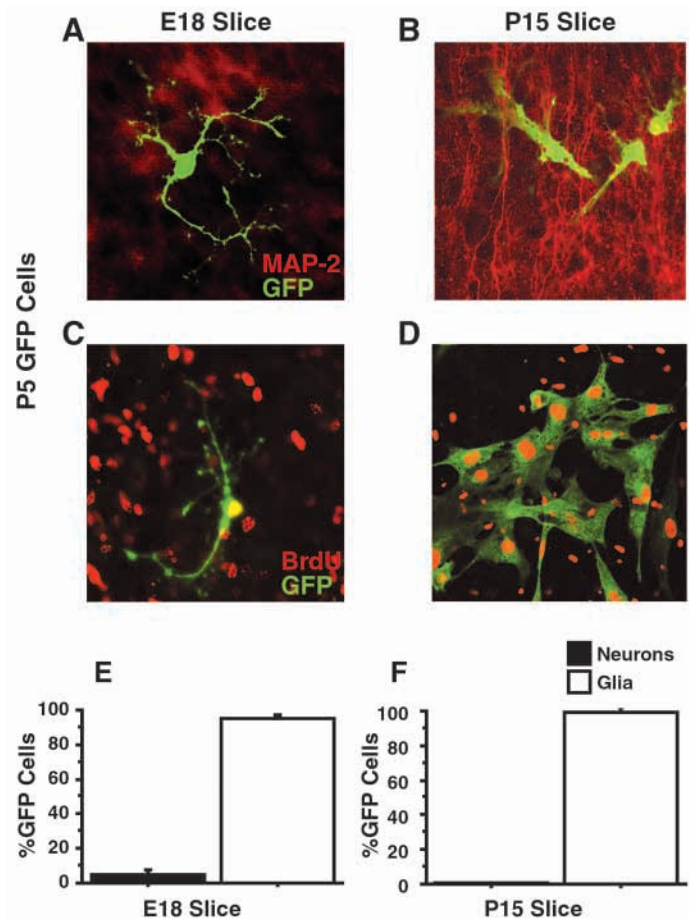


Fig. 3. Postnatal cortical progenitor cells are restricted to a glial cell fate. Mouse P5 GFP cortical cells were isolated from the subventricular zone, dissociated, cultured over rat E18 or P15 cortical slices for 5 days *in vitro*, and then processed for double immunofluorescence using anti-GFP (green channel) and anti-MAP-2 (A,B) or anti-BrdU antibodies (C,D; red channel). (A,B) Examples of GFP+/MAP-2- glia growing on top of an E18 slice (A) and a P15 slice (B). (C,D) Examples of GFP+/BrdU+ glia growing on top of an E18 slice (C) and a P15 slice (D). (E,F) Quantification of GFP cells that differentiated into neurons (filled bars) or glia (open bars) when cultured over E18 or P15 slices. Bar heights represent mean \pm s.e.m. Note that although P5 GFP cells differentiate into glia in the presence of E18 or P15 slices, the cells acquire different morphologies in the two cases, suggesting that developmentally regulated signals may also play a role in specifying glial morphology. The glia that differentiate on top of E18 slices are always multipolar, while those that differentiate on top of P15 slices have a flattened morphology typical of type 2 astrocytes.

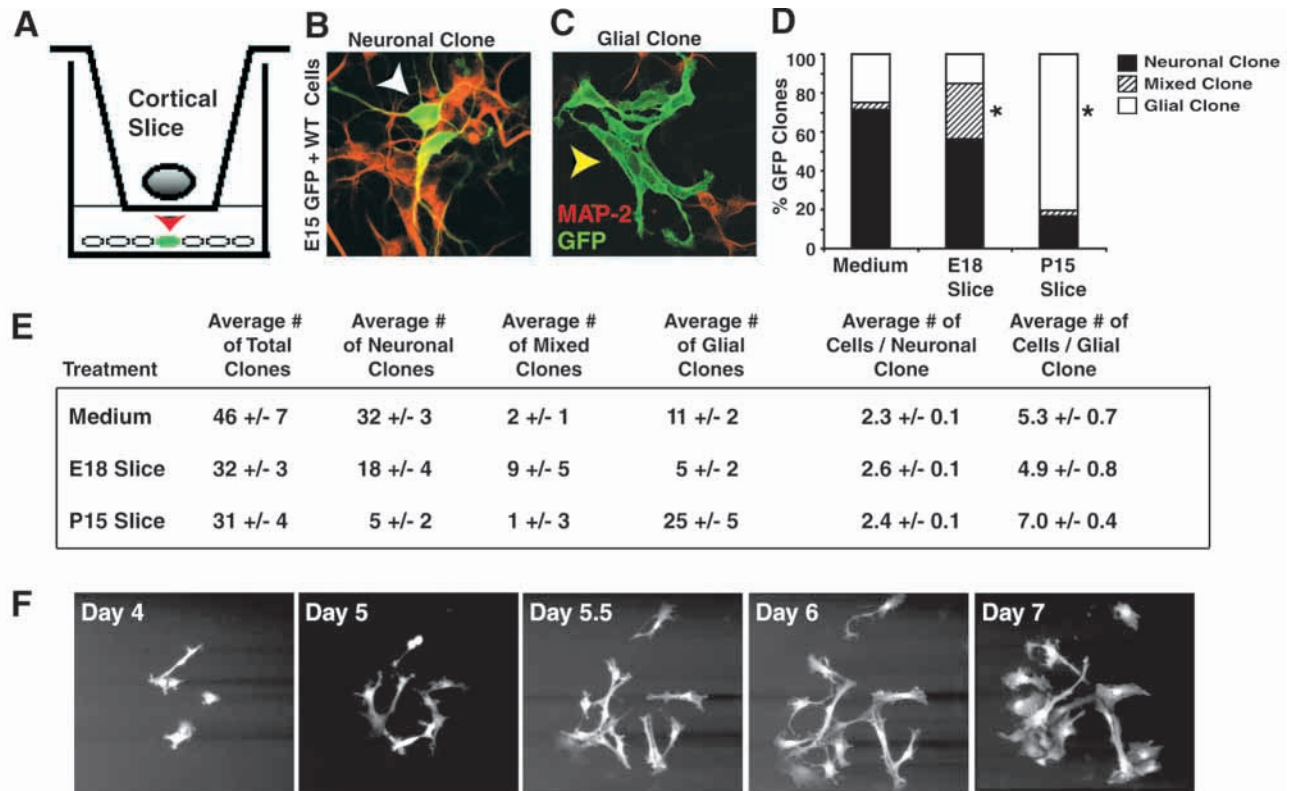
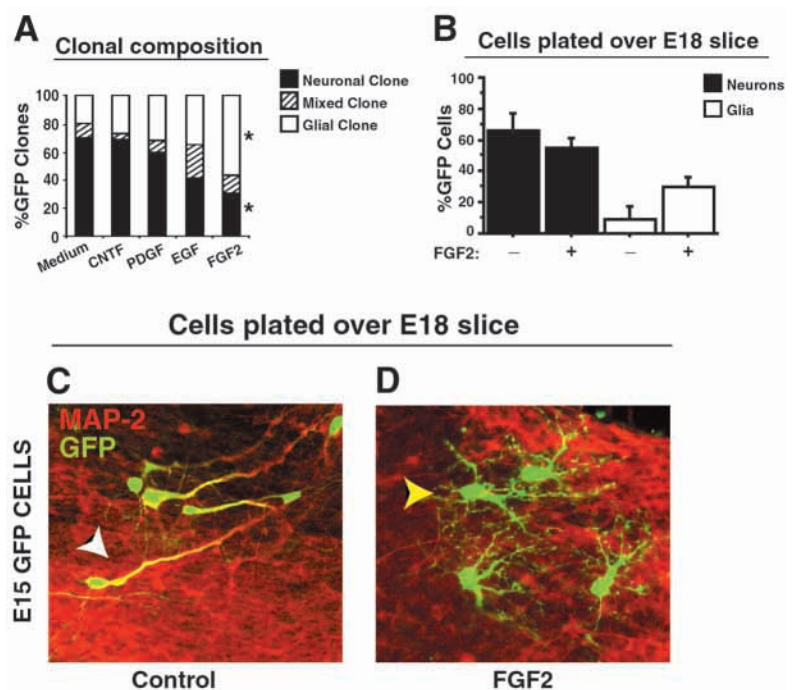


Fig. 4. A diffusible factor present in postnatal cortical slices can induce glial fates. (A) Diagrammatic representation of the assay used to evaluate the influence of cortex-derived diffusible factors on cell fate specification. Dissociated E15 cortical GFP cells were diluted 1:1000 with wild-type E15 cortical cells and plated on glass coverslips underneath membrane inserts containing either rat E18 or P15 cortical slices. After 5 DIV individual clones of GFP+ cells were classified as neuronal (B) or glial (C) by immunofluorescence with anti-GFP (green channel) and anti-MAP-2 (red channel) antibodies. (D) Quantification of the percentage of GFP+ clones that contained only neurons (filled bars), glia (open bars), and both neurons and glia (mixed clones; hatched bars) under indicated experimental conditions. Asterisks indicate statistically significant differences ($P < 0.05$) between the experimental condition and control (medium) for the indicated clonal type. (E) Quantification of the average number of total GFP+ clones, the average number of neuronal, glial and mixed clones, and the average cell number in either neuronal, glial, or mixed clones under indicated experimental conditions (\pm s.e.m.). (F) Time-lapse images of the development of a GFP-positive clone between 4 and 7 DIV.

Fig. 5. FGF-2 induces a cell fate switch in clonal cultures and slice overlay assays. (A) Dissociated E15 cortical GFP cells were diluted 1:1000 with wild-type E15 cortical cells and plated on glass coverslips in medium supplemented with 10 ng/ml CNTF, 10 ng/ml PDGF, 10 ng/ml EGF, or 10 ng/ml FGF2. The percentage of clones that were composed only of neurons (filled bars), only of glia (open bars), and both neurons and glia (mixed clones; hatched bars) are shown as a percentage of the total number of GFP+ clones. Asterisks indicate statistically significant differences ($P < 0.05$) between the experimental condition and control (medium) for the indicated clonal type. (B) Quantification of the number of neurons (filled bars) and glia (open bars) over E18 slices expressed as a percentage of the total number of GFP+ cells on top of the slice under indicated experimental conditions. Bar heights represent mean \pm s.e.m. (C,D) Dissociated E15 GFP cells were cultured over rat E18 cortical slices for 5 DIV in the absence (C) or presence (D) of 50 ng/ml of recombinant FGF2, and then processed for double immunofluorescence with anti-GFP (green channel) and anti-MAP-2 (red channel) antibodies. The GFP cells differentiate into neurons (C: white arrowhead) under normal conditions, and into glia (D: yellow arrowhead) in the presence of FGF2.



majority of the GFP+/BrdU+ cells cultured over E18 slices differentiated into neurons (Fig. 2A,D), the converse was true for GFP+/BrdU+ cells cultured over P15 slices. (Fig. 2C,F). The ability of cortical signals to induce neuronal differentiation of progenitor cells was restricted to the embryonic period, since there were virtually no GFP+/BrdU+ neurons in the cortical slice overlay assays in which E15 GFP cells were plated over P0 or P15 cortical slices (Fig. 2B,C,E,F). Thus, the differentiated fate of embryonic progenitor cells can be influenced by developmentally regulated extracellular signals.

Postnatal cortical progenitors are restricted to glial fates

While the ability of embryonic and postnatal cortical slices to influence the differentiation of cortical progenitors suggests that extracellular signals can regulate neuronal and glial differentiation in the cortex, they do not exclude the possibility that cortical progenitors might also undergo restrictions in cell fate during development. To determine if progenitors in the postnatal cortex are restricted to glial cell fates, we cultured progenitor cells from the subventricular zone (SVZ) of P5 GFP transgenic mice over E18 and P15 cortical slices. In contrast to E15 GFP progenitor cells, which differentiated into neurons or glia depending upon the age of the slice on which they were cultured, P5 GFP cells differentiated exclusively into glial cells when plated over either E18 or P15 cortical slices (Fig. 3A-F). Also, the majority of P5 GFP glial cells growing over both E18 and P15 slices incorporated BrdU (Fig. 3C,D; data not shown). Thus, unlike embryonic progenitors, postnatal cortical progenitor cells are restricted to glial fates.

A developmentally regulated cortical factor can induce a cell fate switch

Developmentally regulated signals may influence the final distribution of neurons and glia by instructively specifying a particular cell fate or by promoting the survival of lineage-restricted progenitors. These alternative cellular mechanisms can be distinguished by examining the effects of extracellular signals on clones derived from single progenitor cells. To study cortical cell fate specification at a clonal level, we cultured E15 GFP cells with a thousand-fold excess of E15 wild-type cells (GFP clonal cultures). The GFP cells in such cultures are well separated from other GFP cells, allowing individual clones of GFP cells to be analyzed.

To evaluate the influence of extracellular signals on clone survival and cell fate specification, we analyzed clone viability and clone composition in cultures growing under control conditions (medium) or underneath E18 or P15 cortical slices (Fig. 4A). Our analysis was restricted to GFP clones of two or more cells (indicating that the cells had undergone at least one round of cell division in culture). To determine if factors produced by E18 or P15 slices differentially affected clone viability, we counted the change in the total number of clones over the culture period under different experimental conditions. In control cultures, at 5 days the number of clones was $75 \pm 5\%$ of the clones present at day 1. In comparison, clone viability at 5 days underneath E18 and P15 was $67.6 \pm 3\%$ and $66.2 \pm 2\%$ respectively. Thus, although there is some loss of clones under all conditions, clone survival rates were comparable under E18 and P15 slices.

To determine the influence of cortex-derived signals on cell

fate, clones were classified as neuronal (clones with only neurons; Fig. 4B), glial (clones with only glia; Fig. 4C) or mixed clones (clones containing both neurons and glia). Analysis of clone composition revealed that factors produced by the cortical slices could exert a major influence on cell fate specification. In serum-free medium, 70% of the clones were neuronal and about 25% were glial, indicating that under control conditions the majority of progenitors give rise to neuronal clones (Fig. 4D). Under E18 slices, there was an increase in the number of mixed clones and a small decrease in the fraction of both neuronal and glial clones (Fig. 4D). In striking contrast, under P15 slices only about 15% of the clones were neuronal and 80% of the clones were glial (Fig. 4D). Thus, a diffusible factor produced by P15 cortex can induce cortical progenitors to adopt glial fates.

Quantification of the total number of each class of clones in different experimental conditions indicated that there was an absolute increase in the number of glial clones when cells were grown below P15 slices (Fig. 4E). This observation, together with the fact that clone viability is comparable under E18 and P15 slices, strongly suggests that a factor produced by the postnatal cortex exerts an instructive influence on cortical progenitors to induce glial fates. At the same time the drop in both the absolute and relative number of neuronal clones under P15 slices suggests that the postnatal factor inhibits neuronal fates. It is also noteworthy that E18 slices induce a 5-fold increase in mixed clones. Since mixed clones arise from multipotent progenitors, this result suggests that E18 cortex may provide a signal that is important in maintaining a stem cell phenotype.

To determine if the embryonic or postnatal cortex exerted a major effect on cell proliferation within clones, we also counted the total number of cells per clone under different experimental conditions. As shown in Fig. 4E the presence of E18 or P15 cortical slices did not significantly affect neuronal clone size. However, culturing cells underneath P15 slices did lead to a small increase in glial clone size, suggesting that the P15 cortex might also produce a mitogen for glial cells.

The strength of our clonal analysis rests on our ability to identify groups of cells as being clonally derived, and the identification of an isolated group of cells as a clone requires that there be limited migration of cells away from the clone. To determine the extent of migration of GFP cells away from a clone, we carried out time-lapse imaging of identified GFP-positive clones over periods up to 3 days in culture. As expected, clone size increased during these observations as a result of cell division, but there was little, if any, migration. An example of a clone imaged between 4 and 7 days *in vitro* is shown in Fig. 4F and represents the spatially restricted expansion of a typical clone. We also measured the average clone diameter and average distance between nearest neighbor clones to determine the likelihood of clonal overlap. The average clonal diameter ($87 \pm 6 \mu\text{m}$) was much smaller than the distance between nearest neighbor clones ($410 \pm 26 \mu\text{m}$), indicating that the probability of clonal overlap was extremely small.

FGF2 and CNTF regulate distinct aspects of glial cell differentiation

While the clonal analysis indicates that the postnatal cortex produces a signal that can induce glial fates, it does not address

whether glial fate specification and terminal differentiation can be independently regulated. One possibility is that once a cell receives a cell fate specification signal, it initiates a cascade of biochemical changes that inevitably leads to terminal differentiation. Alternatively, fate specification and terminal differentiation may be under the control of distinct extracellular signals. One way to distinguish between these possibilities is to ask if purified growth factors that induce glial fates also induce terminal differentiation. We therefore decided to examine the effects of a number of putative glial differentiation signals on cell fate specification and terminal differentiation.

To determine if potential gliogenic signals can induce a cell fate switch in cortical progenitor cells, we cultured E15 GFP cortical progenitor cells in clonal culture in the presence of CNTF, PDGF, EGF, or FGF2 for 5 days, and then analyzed clonal composition. In medium alone, 75% of the clones were neuronal, 10% were mixed, and 15% were glial (Fig. 5). Of the various factors tested, only FGF2 was effective in inducing a cell fate switch comparable to that induced by postnatal cortex (cf. Fig. 4D). In 10 ng/ml FGF2, there was a dramatic reduction in the number of neuronal clones (to 25%), and an increase in the number of glial clones (to 60%). This FGF2 effect is similar to that reported by Temple and colleagues using clonal analysis of isolated cortical progenitors (Qian et al., 1997). In contrast, CNTF, EGF and PDGF did not affect the percentage of glial clones, although EGF led to an increase in mixed clones. The EGF effect on mixed clones is similar to the effect of E18 slices on mixed clones (cf. Fig. 4D), suggesting that EGF may be a component of the E18 activity. Analysis of clone composition and number showed that the shift in clone composition induced by FGF2 cannot be accounted for by selection (data not shown), indicating that FGF2 instructively induces glial fates.

To determine if FGF2 could induce glial fates in a more physiological context, we examined the effect of FGF2 on E15 GFP progenitor cells plated over E18 slices. When E15 GFP cells were cultured over untreated E18 cortical slices, about 65% differentiated into neurons and less than 10% differentiated into glia (Fig. 5B,C). In contrast, when cortical progenitor cells were cultured on E18 slices pre-treated with FGF2, there was a decrease in the number of neurons and over a two-fold increase in the number of glial cells (Fig. 5B-D). This gliogenic effect of FGF2 on cells growing over cortical slices is consistent with a role for this factor in glial fate specification. However, even in the presence of FGF2 the percentage of glial cells is less than that seen with E15 cells plated over P15 slices, indicating that the postnatal cortex contains gliogenic signal in addition to FGF2.

Finally, we examined the effect of developmentally regulated extracellular signals on the terminal differentiation of glial cells. Very few of the cells expressed oligodendrocyte markers in our cultures, and therefore our analysis was restricted to an analysis of extracellular signals on astrocytic differentiation. To determine if developmentally regulated signals regulate astrocyte differentiation, we analyzed expression of the astrocyte marker GFAP in E15 GFP cells that had been plated over E18 or P15 slices but that had not differentiated into neurons (GFP+/MAP-2- cells). As shown in Fig. 6A-C, after 10 days in culture only 25% of the E15 GFP+/MAP-2- cells plated over E18 slices had differentiated into GFAP+ astrocytes, while the remaining 75% remained

GFAP negative, indicating that they had not undergone terminal astrocytic differentiation. In contrast, over 70% of the E15 GFP+/MAP-2- cells plated over P15 cortical slices differentiated into GFAP+ astrocytes (Fig. 6A-C). Thus the terminal differentiation of cortical progenitor cells into GFAP+ astrocytes can be regulated by inductive signal that is produced by the postnatal cortex.

To determine if glial fate specification and terminal astrocytic differentiation can be independently regulated, we examined whether extracellular signals that induce glial fate specification would also lead to astrocyte differentiation. To explore this possibility, we cultured E15 GFP cells over E18 cortical slices for 10 days in the presence of the same growth factors that were tested for glial fate inducing activity (Fig. 5A). Under control conditions, only about 20% of the GFP+/MAP-2- cells differentiated into GFAP-positive astrocytes. Surprisingly, FGF2, which was very effective in inducing glial fates, was ineffective in inducing cells to differentiate into GFAP-positive astrocytes (Fig. 6F). Treatment of these cultures with EGF or PDGF also had no effect on astrocytic differentiation, but CNTF treatment induced a dramatic increase in GFAP expression in the GFP+/MAP-2- cell population (Fig. 6D-F). This effect was comparable to the astrocytic differentiation effect of P15 slices (compare Fig. 6C with 6F), suggesting that CNTF may be a component of the cortical signal that regulates astrocyte differentiation. The fact that FGF2 affects clonal composition but not terminal differentiation, and CNTF affects terminal differentiation, but not clonal composition, indicates that cell fate specification and terminal differentiation can be independently regulated. The ability of FGF2 and CNTF to mimic the effects of P15 cortical slices on glial fate specification and astrocytic differentiation further suggests that these factors are likely to be components of the cortical signal that regulate glial fate specification and differentiation during development.

DISCUSSION

We have examined the influence of extracellular signals on the differentiation of cortical progenitor cells into neurons and glia. Our experiments indicate that the differentiation of early cortical progenitors can be strongly influenced by extracellular signals. When these progenitors are plated over cortical slices from the neurogenic period they differentiate into neurons, and when plated over slices from the gliogenic period, they differentiate into glia. In addition we find that the postnatal cortex produces a diffusible extracellular factor that can induce a cell fate switch in cortical progenitor cells. Thus the sequential generation of neurons and glia in the cortex appears to be controlled by developmentally regulated extracellular factors that can induce a cell fate switch followed by terminal differentiation. We also find that FGF2 and CNTF can selectively affect either cell fate specification or terminal differentiation, indicating that these two steps in glial differentiation can be independently controlled.

The observation that postnatal cortical progenitors are restricted to glial fates suggests that there may be a developmental restriction of cell fate in the cortical progenitor population. While the alternative possibility, that postnatal

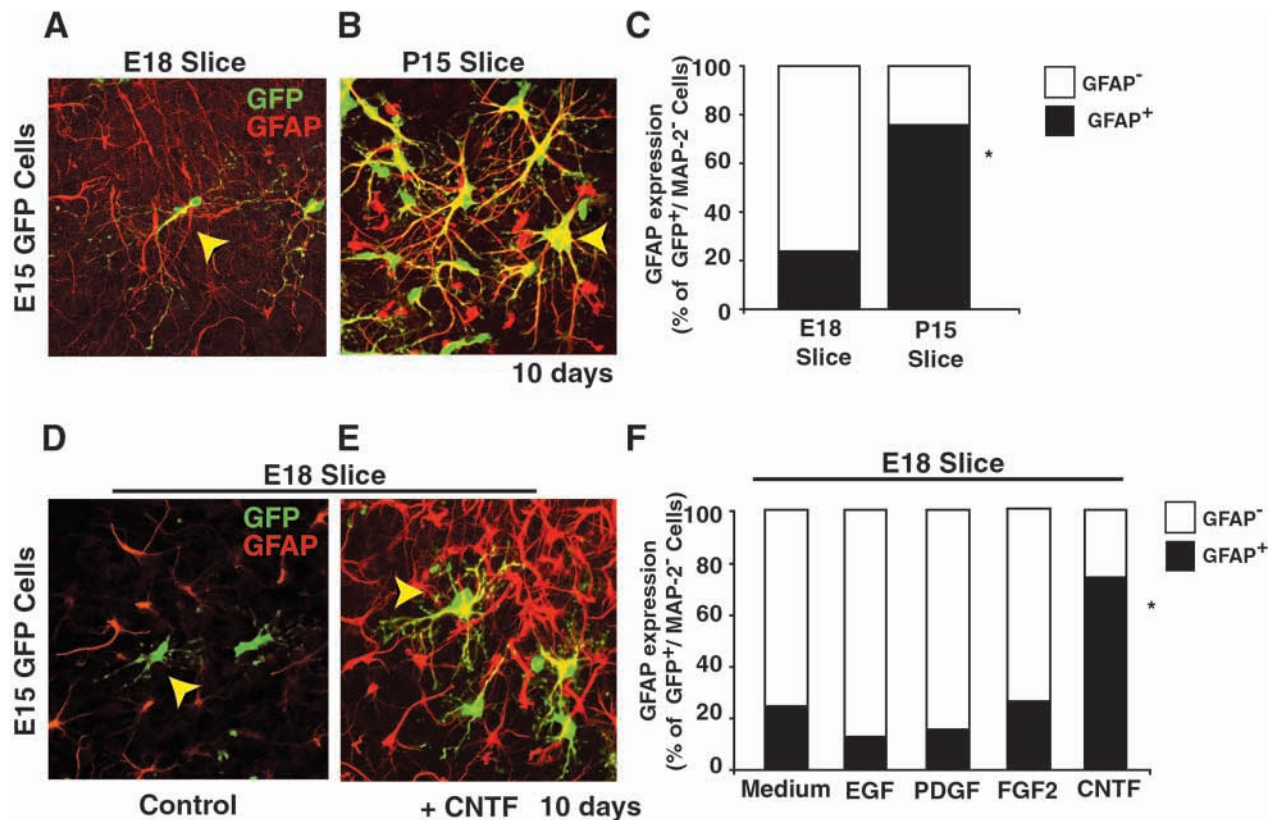


Fig. 6. A signal in postnatal cortical slices and CNTF induce terminal astrocytic differentiation. (A,B) Dissociated E15 GFP cells were cultured over rat E18 or P15 cortical slices for 10 DIV. Examples of E15 GFP⁺/GFAP⁺ cells on top of E18 slices (A), and P15 slices (B) that were processed for double immunofluorescence with antibodies directed against GFP (green channel) and GFAP (red channel). The yellow arrowheads show GFP⁺/GFAP⁺ astrocytes. (C) Quantification of the differentiated fate of GFP⁺/MAP-2⁻ cells cultured for 10 days over E18 and P15 cortical slices. Asterisks indicate statistically significant differences ($P < 0.05$) in the percentage of GFP⁺/GFAP⁺ cells over P15 slices compared to E18 slices. (D-F) Dissociated E15 GFP cells were cultured over rat E18 cortical slices for 10 DIV in the absence (D) or presence (E) of 50 ng/ml of recombinant CNTF, and then processed for double immunofluorescence with anti-GFP (green channel) and anti-GFAP (red channel) antibodies. (F) Quantification of the differentiated fate of GFP⁺/MAP-2⁻ cells cultured for 10 days over E18 and P15 cortical slices in the presence of indicated growth factors, expressed as a percentage of the total number of GFP⁺ cells on top of the slice. Asterisks indicate statistically significant differences ($P < 0.05$) in the percentage of GFP⁺/GFAP⁺ cells over E18 slices in the presence of CNTF compared to control (medium) conditions.

cortical progenitors are not derived from embryonic cortical progenitors, cannot formally be excluded, it is worth considering how cortical progenitors might undergo cell fate restriction during development. Our findings suggest that cell fate restriction may be a consequence of extracellular signals acting on a multipotent progenitor. This possibility is supported by several of our observations. First, we find that embryonic cortical progenitor cells can differentiate into glia when placed onto postnatal cortical slices without first generating neurons. Thus the progenitors do not necessarily generate neurons before generating glia as would be predicted if cells undergo a cell autonomous switch. Second, our experiments on the influence of late cortical slices on clonal composition indicate that a cortical signal can induce progenitors to adopt glial fates at the expense of neuronal fates. Third, we find that there is a developmental increase in an extracellular signal that induces glial cell fates. Together these

observations suggest that an extracellular signal acts on a multipotent progenitor to induce a glial fate. The response of older progenitors placed on embryonic slices suggests that once cortical progenitors adopt a glial fate, they cannot give

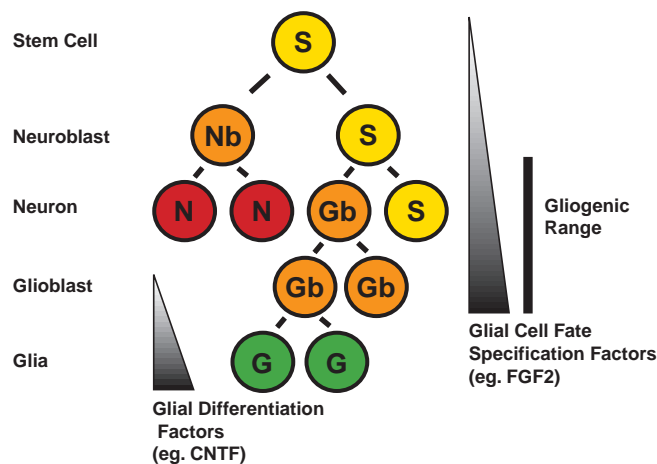


Fig. 7. Model of possible regulation of cell fate decisions and glial differentiation by extracellular factors in the developing cerebral cortex.

rise to neurons even in a neurogenic environment. Such a restriction of progenitor cells to glial fates late in development is similar to a developmental fate restriction in neuronal subtype that has been reported by McConnell and colleagues (McConnell, 1988; Frantz and McConnell, 1996).

Our clonal analysis indicates that a diffusible signal, present in postnatal cortex, can induce glial cell fates. To begin to identify the molecular components of this signal we tested a number of growth factors that are expressed in the developing cortex. The only factor that induced a cell fate switch comparable to that induced by postnatal cortex was FGF2. This adds to the growing evidence that FGF2, which is expressed in the embryonic cortex, can regulate both cell proliferation and cell fate specification in the cortex (Ghosh and Greenberg, 1995; Qian et al., 1997). Injection of FGF2 into the embryonic cortex leads to an increase in the number of neurons and glia, and *fgf2* null mice have reduced numbers of neurons and glia (Dono et al., 1998; Ortega et al., 1998; Vaccarino et al., 1999). These in vivo results have been interpreted as reflecting a mitogenic role for FGF2, but they are also consistent with a role for FGF2 in glial fate specification. It will be of interest to carefully examine the *fgf2* null mice to determine if the effects in vivo are principally due to effects on proliferation, or cell fate specification.

Our data also suggest that factors present in the postnatal cortex act to promote the terminal differentiation of astrocytes. CNTF appears to be a component of the postnatal signal that promotes astrocyte differentiation since CNTF is expressed in the postnatal cortex. Also, adding CNTF to E18 slices induces GFAP expression in both the GFP cells plated over the slice, and in cells within the slice (T. M. and A. G., unpublished observations). These observations add to the evidence that CNTF is an important regulator of astrocyte differentiation (Barres and Raff, 1994; Bonni et al., 1997; Johe et al., 1996; Mi and Barres, 1999). It is striking that CNTF is unable to induce a cell fate switch, although it is very effective in inducing terminal astrocytic differentiation. One possibility is that FGF2 induces CNTF responsiveness in cortical progenitor cells. That would provide for a molecular mechanism by which a cell fate specification signal might induce competence for terminal differentiation in a progenitor cell.

In our slice overlay cultures, cortical progenitor cells typically did not differentiate into oligodendrocytes, the other major class of glial cells. Also, none of the factors we tested was effective in inducing oligodendrocyte differentiation. While this may reflect the existence of other oligodendrocyte differentiation signals (Barres and Raff, 1994; Park et al., 1999), it may also reflect the limitation of the cortical slice overlay assay, which has only been examined for 10 days in vitro. We have previously shown that cortical progenitors grown in FGF2 will differentiate into oligodendrocytes after 10 days in vitro (Ghosh and Greenberg, 1995), and it will be useful to know if cells plated over slices behave similarly in longer term cultures.

Our observations suggest the following model for cell fate specification in the cortex (Fig. 7). During the neurogenic period the cortical progenitor is a multipotent cell that adopts a neuronal fate unless it is exposed to a glial fate-inducing signal. FGF2 can act as a mitogen and a glial fate-inducing factor for this cell, but during the neurogenic period most

progenitors exit the cell cycle and differentiate into neurons because of low levels of extracellular FGF2. A developmental increase in FGF2 signaling (possibly in cooperation with other molecules) induces a glial fate on the remaining dividing progenitors such that all subsequent progeny are restricted to becoming glial cells. These cells, however, do not undergo terminal differentiation unless they are exposed to a second signal that specifically regulates terminal differentiation. CNTF is likely to be an important component of a terminal astrocyte differentiation signal. Such a mechanism, in which developmentally regulated extracellular factors induce multipotent progenitors to adopt a specific cell fate, and subsequently to induce terminal differentiation, might mediate the sequential generation of neurons and glia throughout the nervous system.

We are grateful to Dr Susan McConnell, Dr Alex Kolodkin, and to members of the Ghosh lab for comments on the manuscript. This work was supported by an NIH NRSA award (T. M.) and research grants from NINDS (NS36176), the March of Dimes Foundation (A. G.) and the Pew Scholars Program (A. G.).

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