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Distinct Roles for bFGF and NT-3 in the Regulation of Cortical Neurogenesis

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Summary

To identify molecules that regulate the transition of dividing neuroblasts to terminally differentiated neurons in the CNS, conditions have been developed that allow the neuronal differentiation of cortical precursor cells to be examined in vitro. In these cultures, the proliferation of undifferentiated precursor cells is controlled by basic fibroblast growth factor (bFGF). The proliferative effects of bFGF do not preclude the action of signals that promote differentiation, since addition of neurotrophin-3 (NT-3) antagonizes the proliferative effects of bFGF and enhances neuronal differentiation. In addition, blocking NT-3 function with neutralizing antibodies leads to a marked decrease in the number of differentiated neurons, without affecting the proliferation of cortical precursors or the survival of postmitotic cortical neurons. These observations suggest that bFGF and NT-3, by their distinct effects on cell proliferation and differentiation, are key regulators of neurogenesis in the CNS.

Introduction

A central focus of developmental neurobiology is to understand the role of extracellular signals in regulating differentiation in the nervous system, and over the past several years, this problem has been addressed at several levels. There is considerable evidence that diffusible signals play an important role, even in the earliest stages of nervous system development (reviewed in Jessell and Melton, 1992). For example, the differentiation of neuroectoderm from the surrounding ectoderm can be influenced by mesoderm-derived signals. There has been recent progress in defining the identity of neural inducers, and two molecules, follistatin and noggin, have emerged as likely mesodermal signals that regulate neural differentiation (Lamb et al., 1993; Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou et al., 1994). In a similar vein, but at a later stage of neural development, there is compelling evidence that notocord-derived factors regulate dorsoventral patterning in the spinal cord and may specifically be involved in motoneuron differentiation (Yamada et al., 1993).

At the time of neural induction and neural tube formation, all of the cells in the nervous system are still proliferating, and further differentiation of neural cells into postmitotic neurons and glia must await regionalization of the nervous system into its major subdivisions. Much of our knowledge of the sequence of events involved in neurogenesis derives from studies of the developing cortex, and the major cellular events in this process appear to be conserved elsewhere in the CNS. At the time of regionalization, the cortex consists of a pseudostratified neuroepithelium, which contains proliferating precursor cells. As postmitotic neurons are generated, they migrate away from the neuroepithelium (ventricular zone) and form the cortical plate immediately below the pial surface. In the rat cortex, neurogenesis continues from about embryonic day 13 (E13; E0 is day of mating) to E19 (Bayer and Altman, 1990). This period is followed by the appearance of oligodendrocytes and astrocytes, which are generated during early postnatal development.

Although the cellular events accompanying neurogenesis in the cortex have been described extensively, the underlying cellular and molecular regulatory events are not well understood. The prevailing views of control of neurogenesis are, in fact, based largely on studies of neural crest–derived cells (reviewed in Anderson, 1989; Patterson, 1990). Many of the early experiments dealing with the regulation of differentiation by extracellular signals were performed on the PC12 cell line, which adopts a sympathetic neuron-like phenotype upon exposure to nerve growth factor (NGF) and basic fibroblast growth factor (bFGF; Greene and Tischler, 1976). Subsequently, the issue of regulation of differentiation has been examined in primary cultures of precursor cells. Studies on the development of sympathetic neurons in vitro indicate that these neurons arise from a bipotential sympathoadrenal precursor cell whose differentiated fate can be regulated by extracellular signals. Whereas in the presence of glucocorticoids these cells assume a chromaffin cell fate, bFGF and NGF induce some of these cells to differentiate into sympathetic neurons (Anderson and Axel, 1986). It appears that bFGF initiates neuronal differentiation of sympathoadrenal precursors, and NGF promotes their long-term survival (Stemple et al., 1988). Experiments on an immortalized sympathoadrenal progenitor cell line indicate that ciliary neurotrophic factor (CNTF), FGF, and NGF can cooperatively promote the differentiation of these cells (Ip et al., 1994). Recently, it has also been demonstrated that brain-derived neurotrophic factor (BDNF) promotes the differentiation of neural crest cells along a sensory neuron lineage (Sieber-Blum, 1991), and that BDNF and neurotrophin-3 (NT-3) promote the maturation of embryonic chick sensory neurons in vitro (Wright et al., 1992), suggesting that these factors may be important regulators of neurogenesis in the PNS.

Analogy with neurogenesis in peripheral nervous system suggests that growth factors are likely to be involved in regulating central neurogenesis as well (McKay, 1989).

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Indeed, it has been demonstrated that bFGF is a potent mitogen for certain CNS populations (Gensburger et al., 1987; Cattaneo and McKay, 1990; Ray et al., 1993; Ray and Gage, 1994; Kilpatrick and Bartlet, 1993), and it appears that bFGF and NGF cooperatively promote the proliferation and differentiation of embryonic striatal neurons (Cattaneo and McKay, 1990). It has also been demonstrated that epidermal growth factor, FGF, and transforming growth factor α can influence the proliferation of retinal neuroepithelial cells (Anchan et al., 1991; Lillien and Cepko, 1992). These effects of bFGF are in contrast to the differentiation-promoting effects of bFGF on sympathoadrenal precursor cells and PC12 cells (both derived from the neural crest) and underscore the point that the role of specific factors in regulating CNS neurogenesis cannot be simply inferred from observations in the PNS.

In the last several years, a number of new growth factors have been identified, and their expression has been localized to the developing CNS. Most notable among them are the NGF-related molecules BDNF, NT-3, and NT-4/5 (referred to as NT-4 in this study), which are collectively called the neurotrophins (Leibrock et al., 1989; Hohn et al., 1990; Maisonpierre et al., 1990a; Berkemeier et al., 1991; Hallbook et al., 1991). The expression of the neurotrophins as well as their receptors suggests that they may be involved in regulating aspects of early CNS development. In particular, NT-3 and BDNF are expressed in the embryonic cortex (Maisonpierre et al., 1990b), and their receptors are present in the developing ventricular zone (Tessarollo et al., 1993; Allendoerfer et al., 1994; Escandon et al., 1994; Lamballe et al., 1994). These and related observations (reviewed in Chao, 1992) support the possibility that neurotrophins may regulate proliferation and differentiation in the CNS.

The direct assessment of the role of neurotrophins and other growth factors in regulating CNS neurogenesis under controlled conditions requires the development of appropriate in vitro systems in which the proliferation and differentiation of CNS precursor cells can be examined (McKay, 1989; Cataneo and McKay, 1990). Toward this goal, and specifically to address the issue of cortical neurogenesis, we have developed culture conditions in which cells from the cerebral cortex are cultured at the onset of neurogenesis (E13–14) and in which neurons and glia are generated in appropriate developmental sequence. Our focus in this study has been to evaluate the role of extracellular signals in regulating the early development of cortical neurons, and we report on our findings that bFGF and NT-3 strongly influence cortical neurogenesis by regulating the proliferation and differentiation of cortical precursor cells.

Results

Characterization of an In Vitro System to Study Proliferation and Differentiation of Embryonic Cortical Neurons

In the rat, cortical neurons are generated in the ventricular zone between E13 and E19. The newly postmitotic neurons migrate away from the ventricular zone, and once they reach the cortical plate, they undergo morphologic differentiation and extend axons and dendrites. The transition of neural precursor cells to differentiated neurons is also accompanied by antigenic changes. Whereas the precursor cells are immunoreactive for the intermediate filament nestin (Lendahl et al., 1990) the postmitotic neurons do not express nestin and instead express markers specific for differentiated neurons such as microtubule-associated protein 2 (MAP2; Matus et al., 1981) and TuJ1 (Geisert and Frankfurter, 1989). To examine the interactions that mediate cortical neurogenesis, we developed an in vitro system that would allow us to examine the proliferation and differentiation of embryonic cortical neurons under defined conditions. The development of cells in culture was characterized by various criteria, including the expression of the development stage–specific antigens nestin, TuJ1, and MAP2.

Cultures were prepared from E13–14 animals (E14 cultures), a time when the first postmitotic neurons of the cortex are beginning to differentiate (see Experimental Procedures for details). The cerebral hemisphere was dissected and gently triturated with a fire-polished glass pipette, so that most of the cortex was reduced to clusters of 5–20 cells. The cells were then plated out in an L15 serum-free medium (L15-SFM) at a density of 100 clusters per 16 mm well. To characterize the developmental stages of the cells at time of plating, cells were fixed 4–6 hr after plating and immunostained with antibodies against various neuronal and glial markers. As expected from the developmental time at which the cells are isolated, <5% of the cells expressed markers for differentiated neurons (TuJ1, MAP2) or differentiated glial cells (glial fibrillary acidic protein, galactorcerebroside; data not shown). However, over 95% of the cells were nestin immunoreactive (Figure 1A), indicating that at the time of plating, most of these clusters consisted of ventricular zone cells that had not yet differentiated.

Serial examinations of these cultures over several days revealed a striking difference in behavior between precursors that had been dissociated into single cells and those that had remained in a cluster during plating. The singly dissociated cells (>95%) either immediately stopped dividing or divided only once in culture before becoming postmitotic (Figure 2A). Notably, these singly dissociated cells survived and extended processes in the absence of exogenously added growth factors and expressed neuronal proteins such as MAP2, indicating that they had undergone neuronal differentiation (Figure 2B). In contrast, cell number in the clusters increased over the first few days in culture, indicating that the precursors in the clusters continued to proliferate in vitro. The possibility that cells were undergoing proliferation and neuronal differentiation in vitro was examined more definitively by immunocytochemical methods. Bromodeoxyuridine (BrdU) labeling of these cultures on the day of plating confirmed that about 90% of the cells initially proliferated as determined by nuclear BrdU incorporation (Figure 1B). However, over the first 3–4 days in culture, there was a gradual decrease in the fraction of cells that incorporated BrdU and a correspond-

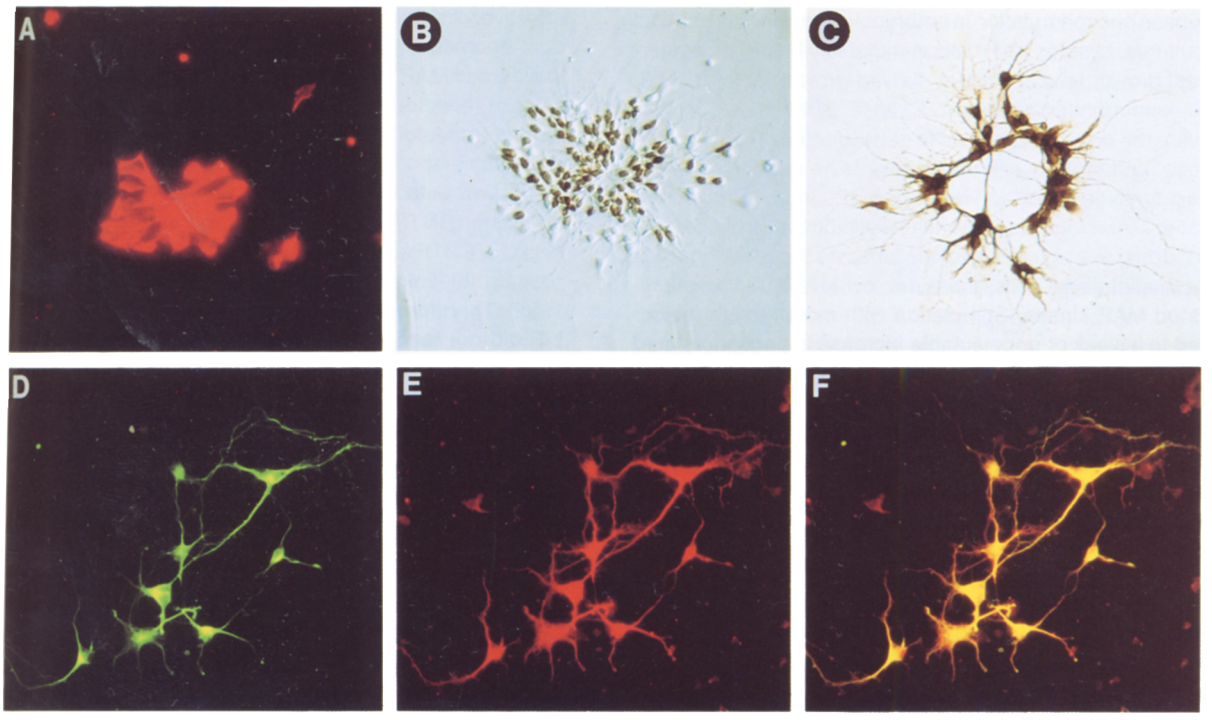


Figure 1. Characterization of E14 Cortex Cultures Growing in the Absence of Exogenous Growth Factors

(A) Immunostaining of a cluster of cells at E14 with a rabbit anti-nestin antibody after 4 hr in culture.
(B) Immunostaining of a cluster of cells at E14 and 1 DIV with an anti-BrdU antibody after being labeled with BrdU on the day of culture.
(C) Immunostaining of a cluster of cells at E14 and 5 DIV with an anti-MAP2 antibody.
(D–F) Anti-TuJ1 (visualized in [D] with a fluorescein-conjugated secondary antibody) and anti-MAP2 (visualized in [E] with a Texas Red–conjugated secondary antibody) label identical populations of cells with neuronal morphologies (double exposure shown in [F]) at E14 and 5 DIV.

ing increase in cells with clear neuronal morphology. By 5 days in vitro (5 DIV), BrdU incorporation was sharply reduced (to <5%), and the clusters were dominated by MAP2-positive neurons (>90%; Figure 1C). The neuronal differentiation of the cells in culture was also confirmed by immunostaining cells for a second neuron-specific marker, TuJ1, which in our cultures labeled a population of cells that were also MAP2 positive (Figures 1D–1F). As shown in Figures 1C–1F, many of the MAP2- and TuJ1-positive cells had extensive dendritic trees typical of morphologically differentiated neurons. Therefore, over the first few days in culture, there was a marked shift in the distribution of proliferating and differentiated cells in these cultures; whereas at the time of plating, over 90% of the cells were nestin positive and incorporate BrdU, by 5 DIV, the proliferating cells were almost entirely replaced by morphologically differentiated MAP2- and TuJ1-positive neurons. These observations indicated that the signals that regulate proliferation and differentiation of embryonic cortical neurons continue to function in these cultures and prompted us to consider the possibility that growth factors are important regulators of neurogenesis under these culture conditions.

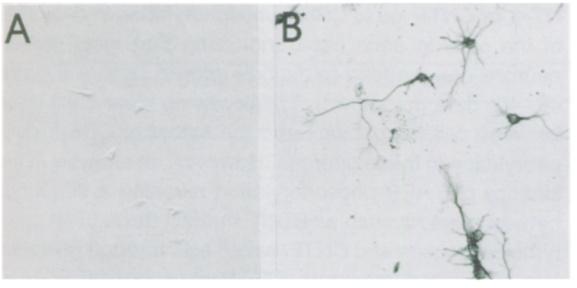


Figure 2. Singly Dissociated E14 Cells Adopt Neuronal Phenotypes In Vitro

(A) Cells isolated from E14 cortex were dissociated and cultured for 24 hr in the presence of BrdU. Subsequent immunostaining with anti-BrdU indicates that in contrast to cells growing in clusters that incorporate BrdU (see Figure 1B), most singly dissociated cells do not incorporate BrdU. (B) Immunostaining of singly dissociated E14 cells after 5 DIV with anti-MAP2 indicates that most of these cells adopt neuronal morphologies and express MAP2, a neuron-specific protein.

Multiple Growth Factors Activate Intracellular Signaling in Developing Cortical Neurons

Growth factor stimulation of receptor tyrosine kinases leads to the activation of several intracellular signaling molecules. Prominent among them is MAP kinase, which is phosphorylated on tyrosine and threonine in response to a variety of extracellular signals (Boulton et al., 1991) and therefore can serve as a useful indicator of functional growth factor signaling pathways. To determine whether developing cortical neurons had signal-transducing growth factor receptors, we examined the ability of several growth factors (known to be expressed in the CNS) to induce MAP

kinase phosphorylation in embryonic and neonatal cortical cultures. Lysates from cells stimulated with bFGF, epidermal growth factor, platelet-derived growth factor, CNTF, leukemia inhibitory factor (LIF), NGF, BDNF, NT-3, or NT-4 were run on a polyacrylamide gel, transferred to nitrocellulose, and probed with phosphotyrosine antibodies to detect tyrosine phosphorylation of MAP kinase. Phosphorylated MAP kinase is present as a distinct band of about 42 kDa on these phosphotyrosine blots. As shown in Figure 3, in unstimulated cells, there are low levels of phosphorylated MAP kinase. Stimulation with most growth factors led to a weak or undetectable increase in phosphorylated MAP kinase in E17 cortical cultures. However, exposure to bFGF, BDNF, NT-3, and NT-4 led to a striking increase in MAP kinase phosphorylation, indicating that receptors for these four factors can signal very effectively in embryonic cortical neurons (Figure 3A). The remaining factors (epidermal growth factor, platelet-derived growth factor, CNTF, LIF, and NGF) were ineffective in leading to significant increases in tyrosine phosphorylation of MAP kinase over a range of concentrations (1–100 ng/ml; data not shown), indicating that this was not simply due to stimulation at suboptimal doses. To determine whether there were developmental changes in growth factor responsiveness in cortical neurons, similar experiments were performed on cultures from neonatal animals. Although the pattern of tyrosine phosphorylation in neonatal cortical cultures was quite similar to that in embryonic cultures, there was one notable difference. Whereas BDNF, NT-3, and NT-4 were still very effective in inducing phosphorylation of MAP kinase, bFGF treatment led only to weak MAP kinase phosphorylation in cultures from neonatal cortex (Figure 3B). These experiments indicate that bFGF, BDNF, NT-3, and NT-4 are effective at activating intracellular signaling in embryonic cortical neurons, and that a developmental change in responsiveness restricts the action of bFGF so that it activates MAP kinase phosphorylation much more effectively in embryonic cortical neurons than in neurons from neonatal cortex.

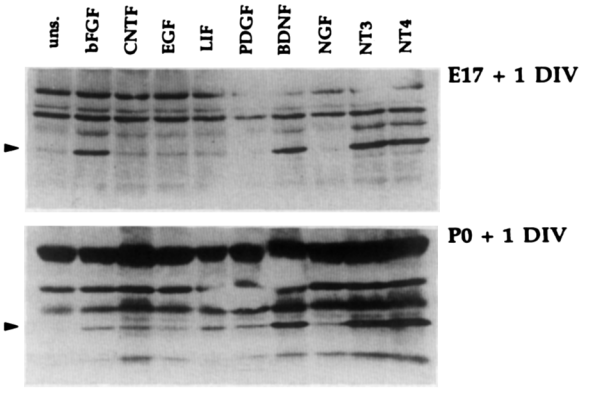


Figure 3. Growth Factor–Induced Tyrosine Phosphorylation in Cortical Neurons

Cultures of E17 (A) and P0 (B) cortex were stimulated at 1 DIV for 5 min with the growth factors indicated (50 ng/ml). Western blots of proteins were probed with phosphotyrosine antibodies. The position of MAP kinase (p42) is indicated by the arrowhead. Whereas BDNF, NT-3, and NT-4 effectively lead to MAP kinase phosphorylation in both E17 and P0 cultures, bFGF is much more effective in signaling in E17 cultures than in P0 cultures.

Whereas MAP kinase phosphorylation is a reliable indicator of receptor activation for growth factors that signal through receptor tyrosine kinases, its role in propagating signals in response to the activation of other receptors is not as well established. Of the growth factors examined, all except CNTF and LIF act through receptor tyrosine kinases. CNTF and LIF act through the activation of glycoprotein 130–linked receptors (Davis et al., 1991; Ip et al., 1992). Therefore, it was possible that although CNTF and LIF did not lead to MAP kinase phosphorylation at the timepoint examined (5 min), they did activate intracellular signaling in these neurons via other pathways. It has previously been shown that activation of various signaling pathways, including activation of the CNTF receptor, leads to the induction of the immediate early gene c-*fos* (Ip et al., 1992). Indeed, we found that stimulation of cortical neurons with CNTF or LIF led to increased expression of c-*fos*, although the level of induction was attenuated compared with stimulation with BDNF, NT-3, NT-4, or bFGF (Figure 4A; data not shown). Together, the results on MAP kinase phosphorylation and c-*fos* induction indicate that bFGF, BDNF, NT-3, NT-4, CNTF, and LIF can activate intracellular signaling pathways in embryonic cortical neurons.

Although MAP kinase phosphorylation and c-*fos* induction are useful indicators of signaling in response to growth factor stimulation, they do not reveal whether these factors target distinct subsets of cortical neurons or whether these neurons can individually respond to multiple factors. To address this issue, we took advantage of the fact that it has been demonstrated recently that many growth factors lead to the activation of the transcription factor cAMP response element–binding protein (CREB), a regulator of c-*fos* transcription (Ginty et al., 1994). CREB activation requires phosphorylation at Ser133, and this event can be detected immunocytochemically using an antibody that specifically recognizes the phosphorylated form of CREB (Ginty et al., 1993). Whereas in unstimulated cortical neurons, less than 5% of the cells showed phosphoCREB immunoreactivity, stimulation with bFGF, BDNF, NT-3, NT-4, or CNTF led to CREB phosphorylation in over 90% of the cells in each case, indicating that most cortical neurons can respond to multiple growth factors (Figures 4B–4D; data not shown). There was no clear correlation between cell morphology and the extent of CREB phosphorylation in these neurons. However, an analysis of the kinetics of CREB phosphorylation revealed a difference between factors such as BDNF that act through receptor tyrosine kinases and CNTF, which acts through glycoprotein 130–linked receptors. CREB phosphorylation in response to bFGF, BDNF, NT-3, or NT-4 peaked by 10 min and remained elevated for several hours; in contrast, there was no detectable effect of CNTF on CREB phosphorylation until about 1 hr, indicating a delayed kinetics of activation in response to CNTF (Table 1). Also, the intensity of phosphoCREB immunoreactivity was significantly greater in response to the neurotrophins than in response to CNTF. These differences in the kinetics and extent of MAP

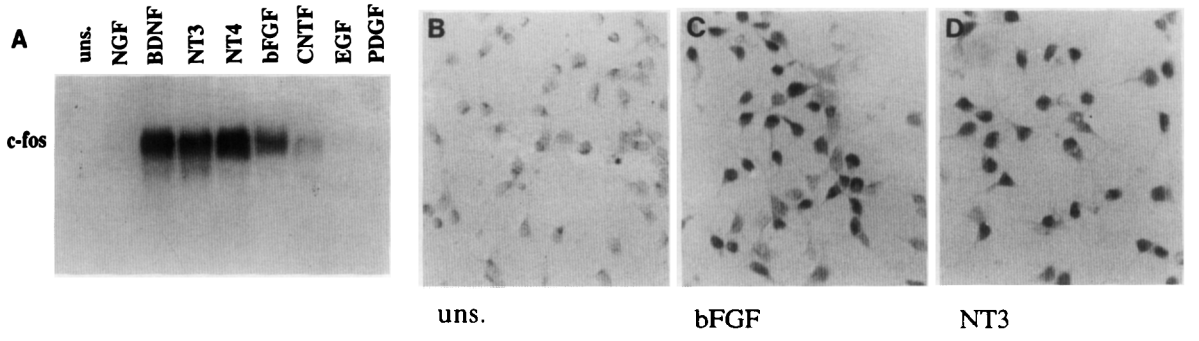


Figure 4. Growth Factor–Induced c-*fos* Expression and CREB Phosphorylation in Cortical Neurons

(A) Induction of c-*fos* expression. Cultures of E17 cortex were stimulated for 60 min with the growth factor indicated (50 ng/ml) before being harvested for RNA isolation. RNA blots were hybridized with a ^{32}P-labeled probe to the coding region of c-*fos*. Of the growth factors examined, BDNF, NT-3, NT-4, bFGF, and CNTF led to detectable increases in c-*fos* expression.

(B-D) Induction of CREB phosphorylation. Cultures of E17 cortex were stimulated for 10 min with the growth factors indicated, and following fixation, the cultures were immunostained with an antibody to the phosphorylated form of CREB. Stimulation with bFGF or NT-3 leads to increased PCREB immunoreactivity in over 90% of the cells.

kinase phosphorylation may reflect differences in the intracellular signaling pathways activated by these different factors that may be relevant to their distinct biological effects.

bFGF Regulates the Proliferation of Embryonic Cortical Neurons

To determine whether the proliferation of cortical cells was regulated by growth factors, cultures were treated with various factors (40 ng/ml) immediately after plating, and their development in vitro was examined over the next several days. To detect proliferating cells specifically, cultures were labeled with BrdU after 4 DIV, and after an additional 24 hr, cells were fixed and processed for BrdU immunocytochemistry. Phase contrast microscopy in these cultures indicated that whereas there was no significant change in cluster size compared with controls when cells were treated with most growth factors, there was a marked increase in cluster size in cultures treated with bFGF (Figures 5A and 5B). This apparent increase in cell proliferation in response to bFGF was confirmed by examining the distribution of BrdU-labeled cells. As shown in Figures 5C and 5D, in the presence of bFGF there was a striking increase in total cell number as well as in the number of cells that had incorporated BrdU. A comparison of the effects of various growth factors clearly indicates that whereas bFGF is very effective at promoting the proliferation of cortical cells, the other growth factors examined do not have such mitogenic activity (Figure 5E).

bFGF promotes the proliferation of cortical cells at concentrations likely to be in the physiologic range. Dose–response studies on both the number of clusters containing BrdU-positive cells (Figure 5F) and the number of BrdU-positive cells per cluster (Figure 5G) indicate that the bFGF response is half maximal between 1 ng/ml and 3 ng/ml. Significantly, however, bFGF led to the proliferation only of cells in clusters and not those that were dissociated as single cells (data not shown). Therefore, the ability of bFGF to promote cell proliferation appears to require cell–cell contact among precursor cells.

To determine whether cells proliferating in the presence of bFGF retain properties of neuronal precursor cells, we examined whether dividing cells continue to express the precursor cell marker nestin. Cultures growing in bFGF for 4 DIV were labeled with BrdU for 24 hr and were subsequently double-labeled with anti-BrdU and anti-nestin antibodies. As shown in Figure 6A, every BrdU-positive cell is also nestin positive. These observations indicate that although several of the growth factors examined can signal effectively in cortical neurons, only bFGF cells promotes the proliferation of nestin-positive cortical precursor cells.

Table 1. Kinetics of CREB Phosphorylation (E17 Ctx)

Factor	10 min	1 hr	4 hr
Uns	–	–	–
NGF	–	–	–
BDNF	++	++	++
NT-3	++	++	++
NT-4	++	++	++
bFGF	++	++	++
CNTF	–	+	+
Epidermal growth factor	–	–	–
Platelet-derived growth factor	–	–	–

Phosphorylation of CREB at Ser133 was detected by immunostaining with an antibody that specifically recognizes the phosphorylated form of CREB. PhosphoCREB immunoreactivity is scored here in terms of relative staining intensity: minus, no detectable staining; single plus sign, weak staining; double plus signs, intense staining.

Cells Undergo Terminal Cell Division and Neuronal and Glial Differentiation in bFGF-Treated Cultures

Serial examination of cultures growing in the presence of bFGF indicated that the proliferation of cells in clusters did not preclude the appearance of morphologically differ-

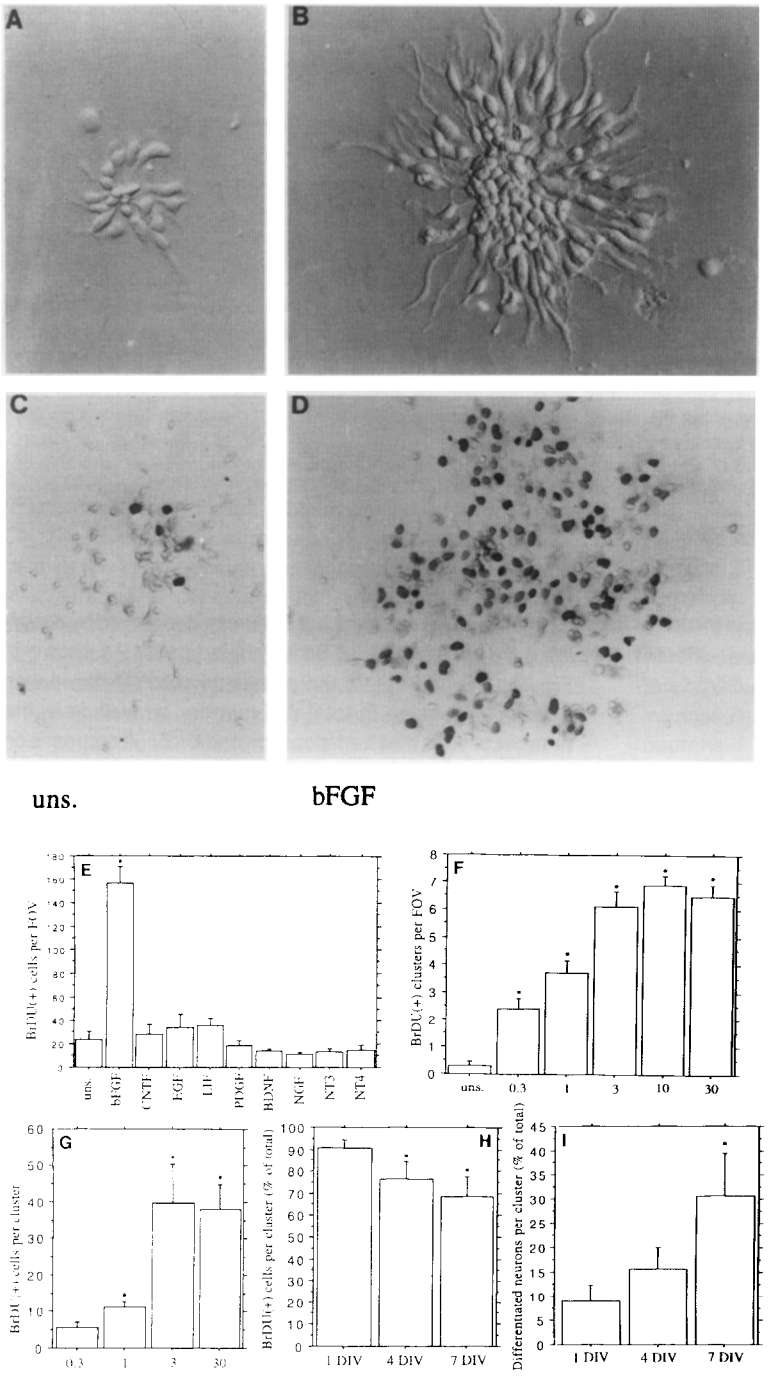


Figure 5. bFGF Promotes the Proliferation of Embryonic Cortical Cells

(A and B) Morphology of E14 cortical cells visualized by phase contrast microscopy at 5 DIV when grown in the absence (A) or presence (B) of 40 ng/ml bFGF.

(C and D) The distribution of BrdU-labeled cells in E14 cortical cultures at 5 DIV (labeled for 24 hr, beginning at 4 DIV) when grown in the absence (C) or presence (D) of 40 ng/ml bFGF.

(E) Quantitative effects of exogenous growth factors on the number of BrdU-positive cells per field of view (FOV). All growth factors were added to a final concentration of 40 ng/ml.

(F) Number of clusters per FOV that include BrdU-positive cells at E14 and 5 DIV as a function of bFGF concentration (indicated in ng/ml).

(G) Number of BrdU-positive cells per cluster at E14 and 5 DIV as a function of bFGF concentrations (indicated in ng/ml).

(H) Percentage of cells that are BrdU positive in bFGF-treated cultures as a function of days in culture.

(I) Percentage of cells that are morphologically differentiated, BrdU-negative neurons in bFGF-treated cultures as a function of days in culture.

entiated neurons. As shown in Figure 5H, although about 90% of the cells growing in the presence of bFGF incorporated BrdU during the first day in culture, by 7 DIV, the fraction of BrdU-positive cells was reduced to about 70%. Correspondingly, there was an increase in the fraction of morphologically differentiated neurons from about 10% to 30% during the first 7 days in culture (Figure 5I).

Since neuronal differentiation in vivo is accompanied by a loss of nestin immunoreactivity, we examined whether morphologically differentiated neurons in culture similarly down-regulated nestin expression and expressed neuronal markers. As shown by double immunofluorescence in Figure 6B, after 5 DIV in the presence of bFGF, cells expressing the neuronal marker TuJ1 no longer expressed the precursor cell marker nestin. The appearance of differentiated neurons in bFGF-treated cultures was also confirmed by double-labeling the cultures with BrdU and MAP2. As shown in Figure 6C, at 5 DIV the cells with neuronal morphology are MAP2 positive and are postmitotic, based on the fact that they do not incorporate BrdU.

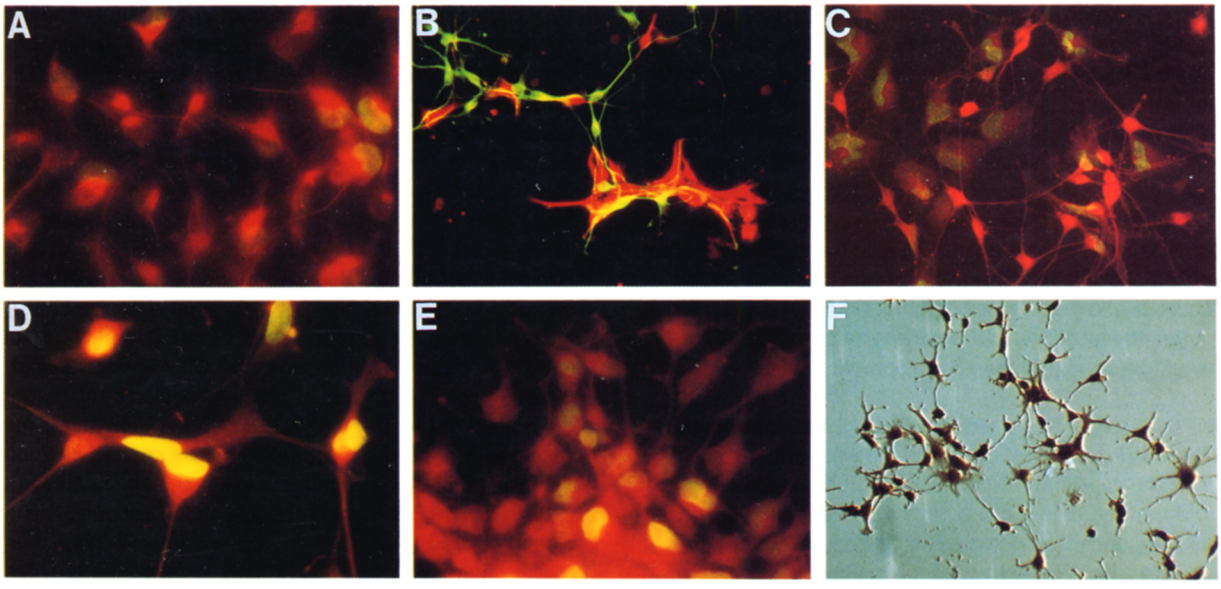


Figure 6. Characterization of E14 Cultures Growing in the Presence of 40 ng/ml bFGF

(A) Double immunofluorescence of cultures at E14 and 5 DIV labeled with anti-nestin (red) and anti-BrdU (green). Cells were labeled with BrdU during the last 24 hr in culture.

(B) Double immunofluorescence of cultures at E14 and 5 DIV labeled with anti-nestin (red) and anti-TuJ1 (green). Note that anti-nestin and anti-TuJ1 label distinct cell populations.

(C) Double immunofluorescence of cultures at E14 and 5 DIV labeled with anti-BrdU (green) and anti-MAP2 (red). Cells were labeled with BrdU during the last 24 hr in culture. Note that the dividing (BrdU positive) cells are a distinct population from the postmitotic neurons (MAP2 positive).

(D) Double immunofluorescence of cultures at E14 and 5 DIV labeled with anti-BrdU (green) and anti-MAP2 (red). Cells were continuously labeled with BrdU beginning at 1 DIV. This experiment indicates that cells that proliferate in culture (BrdU positive) are capable of differentiating into postmitotic neurons (MAP2 positive).

(E) Double immunofluorescence of cultures at E14 and 5DIV labeled with anti-BrdU (green) and anti-Trk (red). Cells were labeled with BrdU during the last 24 hr in culture. Note that proliferating (BrdU-positive) cells express Trk receptors.

(F) Cells labeled with anti-galactocerebroside and visualized by DAB immunocytochemistry at E14 and 12 DIV.

In these photomicrographs, yellow labeling indicates regions of double labeling due to colocalization of Texas Red– and fluorescein-conjugated secondary antibodies.

These observations indicate neurons that differentiate in culture undergo biochemical and cellular transitions typical of cortical neurons that differentiate in vivo.

The increase in cells expressing neuronal markers in bFGF-treated cultures could either be due to up-regulation of neuronal markers by cells that were already postmitotic at the time of plating or due to neuronal differentiation of cells that had undergone at least one round of cell division in the presence of bFGF in vitro. To determine whether cells dividing in the presence of bFGF contributed to the population of postmitotic neurons in our cultures, cultures were labeled with BrdU 1 day after plating, and the fates of BrdU-labeled cells were examined after 5 DIV. Cells that had undergone division in culture were identified by visualizing BrdU-labeled cells, and postmitotic neurons were identified by visualizing MAP2-positive cells. Over 85% of the cells in these cultures were BrdU-positive, indicating that most E14 cells in our cultures divided at least once in vitro (data not shown). More importantly, we found that over 60% of the cells that were immunoreactive for MAP2 were also BrdU positive, indicating that cells that proliferate in bFGF-treated cultures have the capacity to differentiate into neurons (Figure 6D).

The generation of differentiated neurons and glia in vivo is temporally separated. In the rat, all of the neurons are generated during embryonic development, and differentiated oligodendrocytes and astrocytes appear predominantly at postnatal ages. To determine whether bFGF was a mitogen only for neuronal precursor cells or whether glial precursors also proliferated in response to bFGF, we examined the expression of glia-specific antigens in long-term E14 cultures. During the first 7–10 days in culture, less than 2% of the cells had either astrocyte or oligodendrocyte morphologies, and most cells were not immunoreactive for glia-specific markers (galactocerebroside and glial fibrillary acidic protein). Instead, as described above, they were immunoreactive for nestin or MAP2 and TuJ1. However, between 10 and 12 days in culture, the proliferating cells (which, owing to their exponential growth, account for over 90% of the cells by 10 DIV) underwent a striking morphological transformation and adopted a morphology typical of oligodendrocytes. As shown in Figure 6F, over 90% of the nonneuronal cells were intensely immunoreactive for galactocerebroside, indicating that these cells had indeed undergone oligodendrocyte differentiation.

Typically, we do not see the appearance of astrocytes

in these cultures grown in defined media. In serum containing media, however, astrocytes are generated in large numbers, which suggests that the absence of astrocytes in our cultures may reflect the absence of an astrocytic differentiation signal that is present in serum (data not shown). Together, these observations indicate that bFGF promotes the proliferation of cortical precursor cells that are capable of undergoing terminal differentiation into both neurons or oligodendrocytes in the absence of other added factors. Moreover, the sequential appearance of neurons and glia in these cultures is further evidence that under these conditions the cells retain properties that would be typical of their development in vivo.

NT-3 Is a Key Regulator of Cortical Neurogenesis

The observation that cells can undergo terminal cell division and neuronal differentiation in vitro suggests that factors that regulate these events are likely to be produced by cortical cells and can act effectively in culture. Of the growth factors that we found to be effective in signaling in cortical neurons, only bFGF, NT-3, and BDNF have been reported to be expressed in the embryonic cortex. Since bFGF appears to act primarily as a mitogen for neural precursor cells, we considered the possibility that NT-3 or BDNF might regulate the transition of neural precursor cells to differentiated neurons.

The possibility that BDNF or NT-3 may regulate neuronal differentiation would require that their receptors be expressed in the cortical precursor cells. The neurotrophins signal through receptors belonging to the Trk family of receptor tyrosine kinases (reviewed in Glass and Yancopoulos, 1993). Reports on the expression of neurotrophin receptors indicate that Trk receptors are indeed expressed in the ventricular zone during development (Allendoerfer et al., 1994; Escandon et al., 1994). However, it has not been demonstrated conclusively that dividing cells express neurotrophin receptors. To determine whether proliferating cortical precursor cells express neurotrophin receptors in vitro, after 4 DIV we labeled embryonic cultures grown in bFGF with BrdU for 24 hr and then examined the distribution of neurotrophin receptor–expressing cells and proliferating precursor cells by immunostaining with anti-Trk and anti-BrdU antibodies. (The anti-Trk antibody used recognizes all known Trk family receptors: TrkA, TrkB, and TrkC [D. Kaplan, personal communication; A. Ghosh and M. E. Greenberg, unpublished data].) As shown in Figure 6E, virtually all of the cells in the culture are Trk positive, and all BrdU-positive cells also show Trk immunoreactivity, indicating that cells that incorporate BrdU express neurotrophin receptors. The colocalization of BrdU and Trk indicates that proliferating cells express Trk receptors in vitro before undergoing terminal neuronal differentiation.

To address the possibility that endogenous neurotrophins may be involved in regulating neuronal differentiation, we examined the consequences of blocking NT-3 or BDNF function in culture using neutralizing antibodies. We have previously reported on the generation of highly specific antibodies against BDNF and NT-3 (Ghosh et al., 1994). Data regarding specificity of anti-BDNF is already published (Ghosh et al., 1994); experiments demonstrating the specificity of anti-NT-3 are described here. On Western blots of recombinant proteins, anti-NT-3 recognizes NT-3 but not the related neurotrophins BDNF or NGF (Ghosh et al., 1994; J. Carnahan, personal communication), indicating that anti-NT-3 does not nonspecifically cross-react with other neurotrophins. To determine whether anti-NT-3 specifically blocks signaling by NT-3 in cortical neurons, we took advantage of the fact that the expression of the immediate early gene c-*fos* is strongly induced by the neurotrophins BDNF, NT-3, and NT-4. As shown in Figure 7, preadsorption with anti-NT-3 completely inhibited induction of c-*fos* expression by NT-3, but did not affect the ability of BDNF or NT-4 to induce c-*fos* expression in cortical neurons. These experiments indicate that anti-NT-3 recognizes and specifically inhibits signaling by NT-3 without interfering with the actions of even closely related molecules. Moreover, anti-NT-3 does not inhibit differentiation of PC12 cells in response to NGF, indicating that it does not nonspecifically affect neuronal differentiation (data not shown).

As we previously reported, anti-BDNF severely affects the survival of cortical neurons in culture (Ghosh et al., 1994). In preliminary experiments on E14 cultures, we found it difficult to distinguish between the survival effects of BDNF and the role that it may play in promoting neuronal differentiation, since in either case, blocking BDNF function would lead to a decrease in total neuron numbers. To determine whether this would also be a confounding issue in evaluating the role of NT-3, we examined the ef-

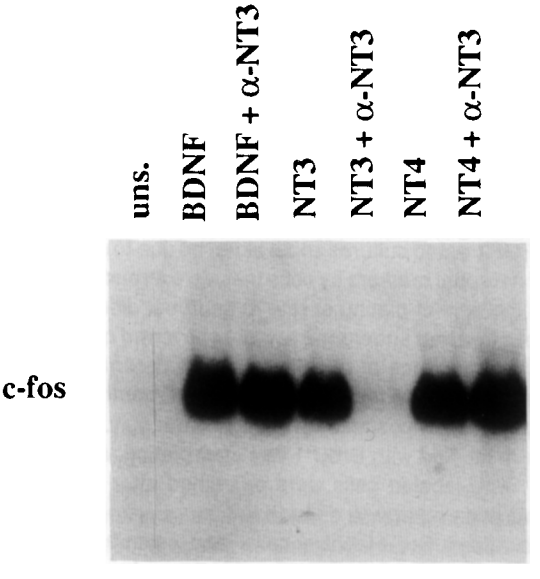
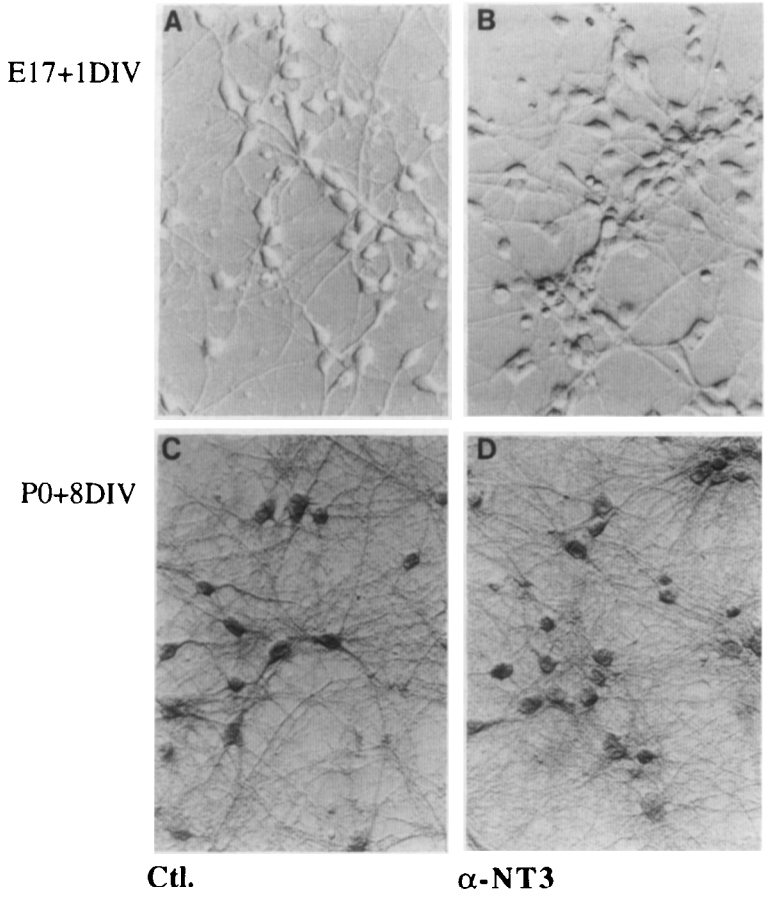


Figure 7. Specificity of Anti-NT-3 Antibodies

Cultures from E17 cortex were stimulated for 1 hr with indicated growth factors (50 ng/ml) or growth factors preadsorbed with anti-NT-3 before being harvested for RNA isolation. RNA blots were hybridized with a ^{32}P-labeled probe to the coding region of the c-*fos* gene. Anti-NT-3 effectively blocks NT-3-induced c-*fos* expression in these cells without interfering with c-*fos* induction by BDNF or NT-4.



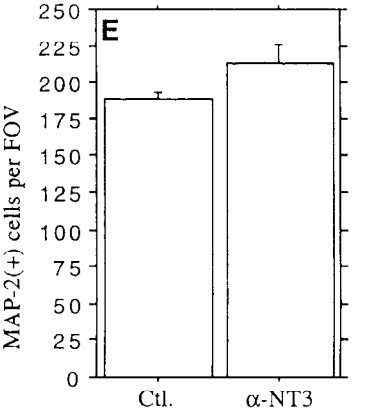


Figure 8. Anti-NT-3 Does Not Affect the Survival of Postmitotic Cortical Neurons in Culture

Cortex cultures at E17–18 and 1 DIV (A and B) or P0 and 8 DIV (C and D) were treated for 48 hr with a control turkey serum (A and C) or anti-NT-3 (B and D). In neither case did anti-NT-3 lead to detectable cell loss.

(A and B) Phase contrast microscopy of live cells.

(C and D) MAP2 immunoreactivity in paraformaldehyde-fixed cells.

(E) Quantitative effects of anti-NT-3 treatment (48 hr) on neuronal survival in E17–18 cortex cultures.

fects of blocking NT-3 function on the survival of postmitotic neurons. Experiments were carried out on E17–18 cultures after 1 DIV or on P0 cultures after 8 DIV. In both cases, cells were treated for 48 hr with a control serum or anti-NT-3. As shown in Figures 8A–8E, in neither case was there a detectable effect of anti-NT-3 on the survival of neurons. Thus, in contrast to anti-BDNF (Ghosh et al., 1994), anti-NT-3 appears not to influence the survival of postmitotic cortical neurons.

Given the expression of neurotrophin receptors on cortical precursor cells and our observation that anti-NT-3 did not significantly affect neuronal survival, we next considered the possibility that endogenous NT-3 might function as a regulator of neuronal differentiation. As shown in Figure 9A, there is normally an increase in the number of differentiated neurons per cluster over the first 7 DIV. To examine the role of NT-3 in neuronal differentiation, E14 cultures growing in the presence of bFGF were treated with a control serum or anti-NT-3. After 6 DIV, cells were labeled with BrdU for 24 hr, and following fixation, cultures were immunostained with anti-MAP2 and anti-BrdU antibodies. As shown in Figure 9B, there was a striking decrease in the number of MAP2-positive neurons in cultures growing in the presence of anti-NT-3. Examination of the cultures indicated that although neuron numbers were dramatically reduced in the presence of anti-NT-3, the morphology of the neurons that were present did not show signs of atrophy (Figures 10A, 10B, 10D, and 10E). This is consistent with the earlier observation that survival of postmitotic neurons in vitro was not affected by anti-NT-3 (see Figure 8) and supports the interpretation that anti-NT-3 was having an effect prior to terminal differentiation. If anti-NT-3 were to influence the proliferation of cortical precursors, it could indirectly lead to a reduction in the number of differentiated neurons. This, however, appears not to be the case, since BrdU labeling experiments indicated that cell proliferation was not affected by anti-NT-3 (Figure 9C; Figures 10C and 10F). Together, these observations strongly suggest that neuronal differentiation in these cultures is dependent upon NT-3 function.

To determine whether levels of endogenous NT-3 were limiting for the induction of neuronal differentiation, we next examined the effects of adding NT-3 to cultures growing in the presence of bFGF. When control and NT-3-treated cultures were examined at 7 DIV, there were no significant differences in the number of differentiated neurons between the two treatments (data not shown). However, when cultures were examined at 4 DIV, there were significantly more differentiated neurons in the NT-3-treated cultures (Figure 9D) than in controls. The fact that

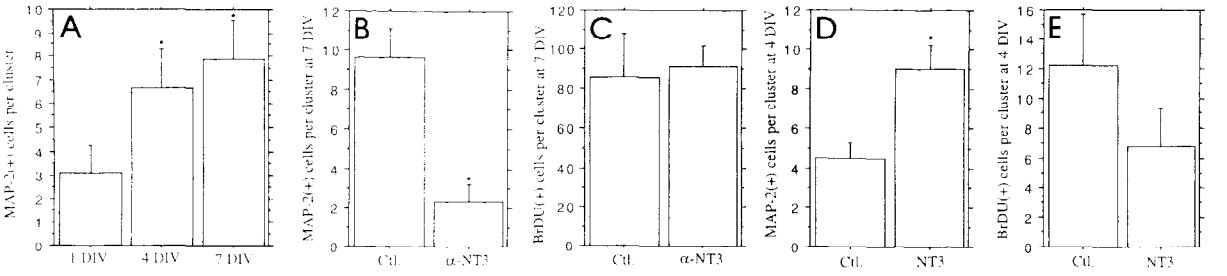


Figure 9. Quantitative Analysis of the Effects of Anti-NT-3 and Exogenously Added NT-3 on Neuronal Differentiation in E14 Cultures

In each of these experiments, cells were grown in the presence of 40 ng/ml bFGF.

(A) Number of MAP2-positive neurons per cluster as a function of days in culture.

(B) Number of MAP2-positive neurons per cluster at 7 DIV in cultures grown in the presence of a control turkey serum (Ctl) or anti-NT-3.

(C) Number of BrdU-positive cells per cluster at 7 DIV in cultures growing in the presence of a control turkey serum (Ctl) or anti-NT-3. Cells were labeled with BrdU during the last 24 hr in culture.

(D) Number of MAP2-positive neurons per cluster at 4 DIV in cultures grown in the absence or presence of added NT-3 (100 ng/ml).

(E) Number of BrdU-positive cells per cluster at 4 DIV in cultures growing in the absence or presence of added NT-3 (100 ng/ml). Cells were labeled with BrdU during the last 24 hr in culture.

added NT-3 has an effect on neuron number at 4 DIV, but not at 7 DIV, suggests that exogenous NT-3 may act to accelerate neuronal differentiation without influencing the eventual number of neurons generated. BrdU labeling experiments indicated that the increase in the number of differentiated neurons at 4 DIV was accompanied by an apparent decrease in cell proliferation (Figure 9E). Therefore, exogenous NT-3 appears to suppress proliferation and promote neuronal differentiation of cortical precursor cells. These experiments complement the NT-3 blocking experiments described above, and together they strongly suggest that NT-3 is a key regulator of differentiation of cortical neurons.

Discussion

To determine the role of extracellular signals in regulating neurogenesis in the developing nervous system, we have examined the effects of peptide growth factors on the proliferation and differentiation of embryonic cortical cells. We find that proliferation of cortical precursor cells is primarily controlled by bFGF, which leads to an expansion of the precursor cell population. Subsequently, the transition of precursor cells to morphologically and biochemically differentiated neurons is regulated by NT-3. These observations indicate that neurogenesis in the CNS is controlled at multiple levels and is strongly influenced by the actions

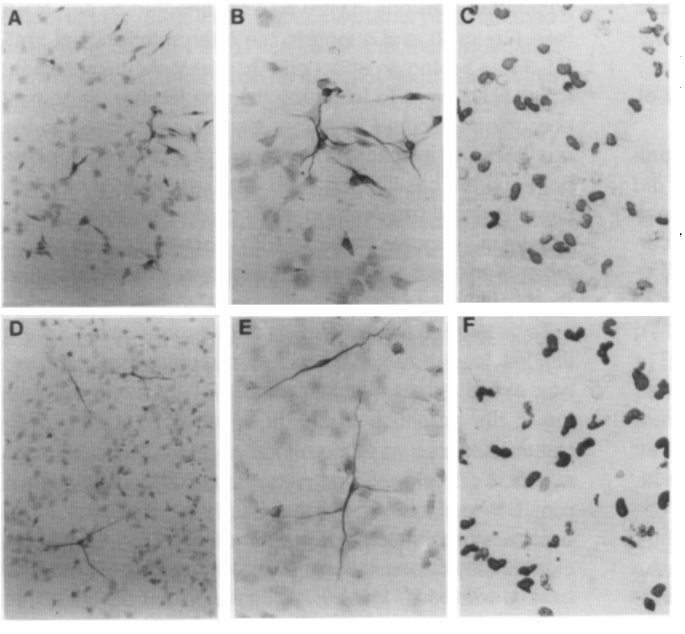


Figure 10. Effects of Anti-NT-3 on Neurogenesis

E14 cortical cells grown in the presence of 40 ng/ml bFGF treated with control turkey serum (A–C) or with anti-NT-3 (D–F). At E14 and 7 DIV, cultures treated with anti-NT-3 show a marked reduction in the number of MAP2-positive neurons (D) compared with controls (A). Higher magnification views (B and E) indicate that anti-NT-3 treatment does not lead to detectable atrophy of the differentiated neurons. BrdU labeling during the last 24 hr in culture (C and F) indicates that anti-NT-3 treatment does not lead to a detectable decrease in cell proliferation.

of bFGF and NT-3, which exert their effects by regulating the proliferation and differentiation of neuronal precursor cells.

Development of Cortical Cells in E14 Cluster Cultures

The analysis of factors that influence CNS neurogenesis was facilitated by developing culture conditions that allowed us to monitor the proliferation and differentiation or embryonic cortical neurons. These cultures differ from more commonly used cortical cultures in three important respects. First, they are prepared from animals at E14, which is at the onset of cortical neurogenesis and allows one to examine the earliest steps of neuronal differentiation. Second, they are grown in a chemically defined media in which the effects of exogenous factors on development can be clearly evaluated. Third, the cultures consist of small clusters of precursor cells that allow for normal cell–cell interactions to regulate development as they would in vivo. This final point is particularly important because we feel that it allows the role of endogenously produced extracellular and intracellular signals that regulate cortical neurogenesis to be evaluated by in vitro perturbation studies.

The development of cells in E14 cultures provides several insights about the role of cell–cell interactions in cortical neurogenesis. First, we find that in the absence of added growth factors, singly dissociated cells from E14 cortex do not undergo cell division, whereas those in clusters proliferate for several days before leaving the cell cycle. This observation suggests that the proliferation of cortical precursor cells requires cell–cell interactions. In light of these observations, it is noteworthy that it has recently been reported that in serum-containing media, groups of cells proliferate more extensively than single cells (Temple and Davis, 1994). Second, the fact that most singly dissociated cells at E14 underwent neuronal differentiation suggests that direct cell–cell interactions are not required for precursors to differentiate into postmitotic neurons. Third, in the absence of added growth factors, we find over 90% of the cells in clusters differentiate into neurons by 5 DIV. This is striking, since precursor cells in our cultures are capable of giving rise to both neurons and glia in the presence of bFGF. One possibility is that at early developmental stages, most precursors are committed to a neuronal lineage. Alternatively, many of the precursors may be bipotential, and bFGF may act to inhibit (but not completely suppress) neuronal differentiation of these cells.

Regulation of Cell Proliferation by bFGF

Our experiments indicate that the proliferation of cortical precursor cells is positively regulated by bFGF. The proliferative effect of bFGF on cortical precursor cells is similar to the effects of bFGF reported previously on other CNS populations (Cataneo and McKay, 1990; Kilpatrick and Bartlet, 1993; Ray and Gage, 1994; Ray et al., 1993; DeHamer et al., 1994) and suggests that bFGF functions widely to promote the proliferation of CNS precursor cells. Our observations on the proliferative effects of bFGF, however, differ from previous observations in some respects that deserve comment. In cultures of striatal precursor cells, Cataneo and McKay reported that whereas bFGF alone was not very effective in promoting cell proliferation, it synergistically acted along with NGF to promote cell division. In our cultures, bFGF functions quite effectively in the absence of NGF, and we see no enhancement of the proliferative response to bFGF in the presence of NGF (A. Ghosh and M. E. Greenberg, unpublished data). In fact, all of our evidence suggests that NGF does not signal (at least through receptor tyrosine kinase activation) in cortical cells. More recently, Ray and Gage reported that high concentrations of bFGF maintain hippocampal neurons in proliferative cells for several weeks without apparent phenotypic changes over time in culture. In contrast, in our experiments, the proliferating precursor cells underwent a fairly synchronous transition to oligodendrocytes after about 10 days in culture. It is possible that these hippocampal cultures do not contain oligodendrocyte precursors, which may suggest a simple explanation for this observed difference. A definitive resolution of these differences will be important for a more complete understanding of control of proliferation of specific CNS precursor populations.

The proliferative effect of bFGF on cortical precursor cells is consistent with the high level of expression of bFGF and FGF receptors in the proliferative (ventricular) zone of the developing cortex (Gonzalez et al., 1990; Powell et al., 1991; Wanaka et al., 1991). Since cells in the E14 cultures continue to proliferate for the first few days in vitro even in the absence of exogenously added bFGF, it is possible that endogenously produced bFGF regulates the proliferation of embryonic cortical neurons in our cultures. In support of this possibility, preliminary experiments suggest that neutralizing antibodies against bFGF (which recognize bFGF and not acidic FGF) suppress the proliferation of E14 cortical neurons (A. Ghosh and M. E. Greenberg, unpublished data).

The high levels of FGF receptor expression on cortical precursor cells suggests that bFGF most likely acts on the precursors directly to promote cell division and therefore acts as a mitogen for these cells. However, since the proliferative effects of bFGF are evident only in cells growing in clusters, it is possible that bFGF induces the precursor cell to release a mitogen that then acts on neighboring cells within a cluster. It is also possible that direct cell–cell interactions are required in addition to bFGF signaling to cause precursor cells to divide. This would be consistent with the observations of Temple and Davis (1994) that membrane-associated factors promote the proliferation of cortical precursor cells. Although at present we cannot distinguish among these possible modes of bFGF action, our experiments provide evidence that bFGF is an important and effective regulator of proliferation of cortical precursor cells.

Our observations also indicate that although bFGF promotes cell proliferation, it does not preclude the action of signals that induce neuronal and glial differentiation. Since

the appearance of differentiated neurons in bFGF-treated cultures precedes the appearance of differentiated oligodendrocytes, an interesting possibility is that the precursor that gives rise to neurons early in development may, over subsequent divisions, gain competence to give rise to oligodendrocytes. Such a change in competence may even be influenced by the neurons that are generated during the early divisions. The recent observations on the existence of multipotent stem cells in the embryonic cortex at least in principle allow for such a mechanism in the generation of oligodendrocytes (Davis and Temple, 1994).

Regulation of Neuronal Differentiation by NT-3

An important finding of this study is that NT-3 can regulate the neuronal differentiation of cortical precursor cells. We show that addition of NT-3 enhances neuronal differentiation, and that blocking endogenous NT-3 function with neutralizing antibodies markedly inhibits neuronal differentiation of cortical precursor cells. Since our conclusions rely in part on the use of neutralizing antibodies, the issue of their specificity deserves comment. The antibodies against NT-3 show remarkable specificity considering the homology among the various neurotrophins. These antibodies recognize recombinant NT-3 but do not cross-react with recombinant NGF, BDNF, or NT-4 on Western blots. They are also effective in specifically blocking NT-3 signaling, since they block NT-3-induced *c-fos* expression in cortical neurons without affecting the induction of *c-fos* in these cells by BDNF or NT-4. Anti-NT-3 antibodies also do not influence NGF differentiation of PC12 cells, indicating that they do not nonspecifically interfere with differentiation. These various specificity controls strongly suggest that observed effects of anti-NT-3 on cortical neurogenesis are indeed due to the disruption of NT-3-mediated events in these cells.

The involvement of NT-3 in regulating neuronal differentiation is consistent with a number of recent observations that suggest that neurotrophins can influence neuronal development prior to terminal differentiation. For example, BDNF, NT-3, and their receptors are expressed in developing sensory and sympathetic ganglia, suggesting that they may play a role in the early development of neural crest–derived cells (Schecterson and Bothwell, 1992). Indeed, it has been shown that NT-3 and BDNF promote the maturation of chick DRG cells in culture (Wright et al., 1992), and that NT-3 promotes the survival of proliferating sympathetic neuroblasts (DiCicco-Bloom et al., 1993; Birren et al., 1993). A recent report also indicates that NT-3 treatment leads to an increase in the number of islet 1–positive cells in quail neural tube cultures, suggesting that NT-3 may be involved in motoneuron differentiation (Averbuch-Heller et al., 1994; Pinco et al., 1993). Both NT-3 and its receptor, TrkC, are expressed in the developing cortex (Maisonpierre et al., 1990b; Tessarollo et al., 1993; Lamballe et al., 1994). Moreover, NT-3 expression in the brain appears to be at its highest during the embryonic period (Maisonpierre et al., 1990b). These observations, together with our finding that NT-3 regulates the neuronal differentiation of cortical precursor cells, suggest that NT-3 is likely to be a key regulator of neurogenesis in both the central and peripheral nervous systems.

Although our experiments indicate that NT-3 can influence neuronal differentiation, it is likely that other factors contribute to the process as well, since animals with targeted disruptions of NT-3 or TrkC do not have a clear deficit in neuronal differentiation (Ernfors et al., 1994; Klein et al., 1994). Although an analysis of cortical development in these animals has not yet been reported, the brain appears to be grossly normal at birth. Although it is possible that other factors can compensate for the loss of NT-3 or TrkC, a detailed analysis of neurogenesis in these animals could be instructive, since they may reveal subtle but important abnormalities. We should also note that in our experiments, even at high concentrations of NT-3 only about 30% of the cells in bFGF-treated cultures differentiated into neurons. Therefore, NT-3 may be capable of inducing neuronal differentiation only in a subset of neuronal precursors. It is not yet clear whether this is due to differences in TrkC expression within precursor cells or whether some other lineage restriction limits the effects of NT-3 on neuronal differentiation.

Though the precise mechanism by which NT-3 regulates differentiation is not clear, our observations suggest that NT-3 acts, at least in part, by promoting the withdrawal of precursor cells from the cell cycle. It has been reported previously that ventricular zone cells express neurotrophin receptors (Tessarollo et al., 1993; Allendoerfer et al., 1994; Lamballe et al., 1994) and that cortical cells are most responsive to neurotrophins during the period of differentiation (Knusel et al., 1994). We show here that dividing precursor cells express Trk receptors and that NT-3 can inhibit the mitogenic effects of bFGF on these cells. These observations indicate that NT-3 inhibits the proliferation of cortical precursor cells and suggest that withdrawal from the cell cycle may be a critical step in neuronal differentiation. Although the mechanism by which NT-3 suppresses proliferation is not known, one possibility is that a critical level of bFGF-induced activation of signaling molecules is required to keep precursor cells dividing and NT-3 antagonizes the proliferative effects of bFGF by inhibiting bFGF-induced intracellular signaling. In this regard, it is noteworthy that we find a developmental decrease in bFGF-induced MAP kinase phosphorylation in cortical neurons that is temporally correlated with cessation of cell proliferation in the developing cortex. We should note, however, that it is not yet known whether the decrease in bFGF-responsiveness in cortical cells occurs prior to or subsequent to withdrawal from the cell cycle.

In summary, based on the expression patterns of various growth factors and their receptors, as well as our findings described here and previously (Ghosh et al., 1994), the following hypothesis emerges regarding growth factor control of early cortical development (Figure 11). The proliferation of cortical precursor cells appears to be regulated by bFGF in the ventricular zone. Subsequently, NT-3 acts to promote cell cycle withdrawal and neuronal differentiation of at least some of the precursor cells, and BDNF and

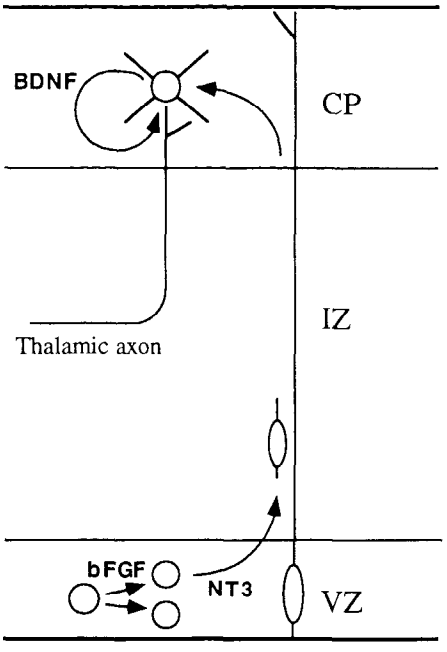


Figure 11. Diagrammatic View of the Developing Cortex Indicating the Events Likely to Be Influenced by Growth Factor Action

The diagram is based on results of this and previous studies. VZ, ventricular zone; IZ, intermediate zone; CP, cortical plate.

bFGF act as trophic factors for the postmitotic neurons (Morrison et al., 1986; Ghosh et al., 1994; A. Ghosh and M. E. Greenberg, unpublished data). Although such a scheme is perhaps oversimplified and does not take into account the likely involvement of other regulatory factors, it conveys the central idea that growth factors regulate specific and distinct aspects of cortical development. A similar framework, in which NT-3 and BDNF regulate distinct aspects of cerebellar development, has recently been proposed (Segal et al., 1992). The exploration of the mechanisms by which specific growth factors regulate these effects should provide us with a clearer understanding of the molecular mechanisms that control CNS development.

Experimental Procedures

Cortical Cell Cultures

E14 Cultures

The cortex from E13–14 animals was dissected in ice-cold HBSS (6.5 g/L glucose) and transferred to culture media (glutamine-free L15 [ICN] with 30 mM glucose, 25.8 mM $NaHCO_3$, 1% N2 supplement [Gibco] with penicillin–streptomycin). The tissue was gently triturated with a fire-polished glass pasteur pipet to yield small clusters of cells (5–20 cells per cluster). Cells were plated onto polylysine–laminin-coated plastic (24 well, Costar) or glass (4 well, Nunc) tissue culture plates at a density of approximately 100 clusters per well.

E17–18, P0 Cultures

The cortex was dissected in HBSS and transferred to an enzyme solution (Papain, 10 U/ml) for 2 × 20 min. Following two washes with trypsin inhibitor, the tissue was transferred to culture media and triturated to yield a single cell suspension. Cells were plated in polylysine–laminin-coated plates at a density of 2×10^6 cells per 60 mm plate or 10^5 cells per well in 24 well plates.

Growth Factors and Antibodies

All growth factors were diluted in PBS and were obtained from the following sources: bFGF and NT-4/5, Amgen; BDNF and NT-3, Amgen-Regeneron Partnership; NGF, David Ginty, HMS; CNTF, Regeneron; platelet derived growth factor, Chuck Stiles, Dana-Farber Cancer Institute; epidermal growth factor, Collaborative Research; LIF, Seth Field, HMS. Anti-NT-3 (103) and anti-BDNF (679–1) (turkey antibodies) were obtained from Dr. J. Carnahan (Amgen).

Treatment of Cultures and Data Analysis

Unless otherwise indicated, growth factors were added at a final concentration of 40 ng/ml. In long-term experiments, factors were supplemented every third day. Anti-NT-3 and anti-BDNF were added at 10 μl/ml media and were not supplemented. In each experiment, cells were treated in duplicate, and each experiment was repeated 2–3 times. For cell counts, data are indicated as mean ± SEM. The statistical significance of variations was evaluated using Student's t test (pairwise comparisons), and statistically significant differences compared with controls ($p < .05$) are indicated with a dot in the figures.

BrdU Labeling and Visualization

BrdU (Boehringer Mannheim) was diluted in sterile dH_2O and added to cultures at a final concentration of 10 μM. For BrdU immunocytochemistry, cells were fixed with 4% paraformaldehyde/4% sucrose in PBS for 15 min and subsequently processed as follows: rinse with PBS, 3 × 5 min, postfix in 70% EtOH, 30 min; rinse with PBS, permeabilize with 0.4% Triton-100 in PBS, 30 min; rinse with PBS, 2N HCl, 10 min, 0.1M NaB_2O_7, 10 min; rinse with PBS, block with 3% BSA, 3% goat serum, 0.3% Triton in PBS, 1 hr; incubate in mouse anti-BrdU (Becton Dickinson) 1:50 in blocking solution O/N at 4°C. For peroxidase-based immunocytochemistry, a Vectastain kit (Vector Labs, CA) was used. For immunofluorescence, cells were washed extensively after primary antibody incubation, incubated in fluorescein-conjugated goat anti-mouse secondary for 1 hr, washed extensively, and viewed under fluorescein epifluorescence optics. All immunofluorescence was performed on cells growing on 4 well Nunc glass culture chambers.

Immunocytochemistry

For all antigens other than BrdU, cells were fixed in 4% paraformaldehyde/4% sucrose in PBS for 15 min, blocked in 3% BSA, 3% goat serum, 0.3% Triton in PBS for 1 hr, and immunostained according to standard procedures. For immunofluorescence, mouse primary antibodies were detected with fluorescein-conjugated goat anti-mouse secondary antibodies, and rabbit primary antibodies were detected using Texas Red–conjugated goat anti-rabbit secondary antibodies. Antibodies were used at the following dilution: anti-nestin (from Dr. Ron McKay, NIH) at 1:50; anti-MAP2 (from Dr. Richard Vallee, Worcester Foundation for Experimental Biology) at 1:5000; anti-TuJ1 (from Dr. Brenda Williams, Boston Biomedical Research Institute) at 1:5000; anti-GFAP (Sigma) at 1:500; anti-galctocerebroside (Sigma) at 1:500; anti-Trk 203 (from Dr. David Kaplan, NCI) at 1:200; anti-phospho CREB (from Dr. David Ginty, HMS) at 1:1000.

Protein Blots

Cells growing in 60 mm plates were stimulated with growth factors for 5 min before being lysed in 100 μl of boiling SDS lysis buffer (160 mM Tris-HCl [pH 6.8], 4% SDS, 30% glycerol, 5% β-mercaptoethanol, 10 mM DTT, 0.02% bromphenol blue). Lysates were loaded on an 8.5% polyacrylamide gel, separated by gel electrophoresis, transferred to nitrocellulose and immunoblotted using phosphotyrosine antibodies (1:1000 4G10 [UBI] and 1:1000 Py20 [ICN]), and visualized using electrochemiluminescence.

RNA Blots

Total cellular RNA from cells growing in 60 mm plates was isolated using the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). RNA was loaded at 15 μg per lane of a 1.4% agarose/formaldehyde gel, transferred to nylon membranes, and hybridized with a ^{32}P-labeled probe to the coding region of the *c-fos* cDNA.

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