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Multiple Factors Control the Proliferation and Differentiation of Rat Early Embryonic (Day 9) Neuroepithelial Cells

Ronghao Li,[1] Wei-Qiang Gao,[2] and Jennie P. Mather[1]

[1]*Cell Biology and* [2]*Neuroscience, Genentech, South San Francisco, CA*

The proliferation and differentiation of neural precursor cells is largely controlled by environmental factors. By providing the factors that favor the proliferation or suppress the differentiation of this cell type, we isolated and expanded an early neuroepithelial predifferentiated cell type from E9 rat neural plate in serum-free medium. This has led to the establishment of a neural epithelial precursor (NEP) cell line. The NEP cell's properties are substantially different from those of cell lines previously derived from neural tissue at later stages of development. Initial selection and survival of this cell type requires a factor secreted by an embryonic Schwann (nrESC) cell line. Continued passage of these cells requires cell–cell contact for both survival and growth. Neural cell differentiation can be induced in this nestin positive precursor cell line by bFGF and forskolin. General neuronal markers, as well as cortical neuron-specific protein kinase C isozyme, and accumulation of glutamate and aspartate were induced in most cells. Choline acetyl-transferase was also induced in a small number of cells. When implanted into neonatal rat brain, the NEP cell line gave rise to several distinct neuronal and glial phenotypes in different regions of the brain including cerebellar cortex and hippocampus.

Key Words: Neural plate; neuroepithelial cell; differentiation; bFGF; serum-free culture; nestin.

Introduction

The mammalian central nervous system is developmentally derived from neuroepithelial cells in the neural plate. These neuroepithelial cells grow and differentiate in response to, as yet unidentified, signals. The ability to isolate and maintain particular neural precursor cells in vitro is crucial to the identification of these factors. Since many types of neuroepithelial cells cultured in vitro tend to differentiate spontaneously, long term culture of neuroepithelial cells has relied on viral or oncogene transformation that, it was hoped, might halt development at specific stages of differentiation. This approach has resulted in the establishment of several immortalized, or conditionally immortalized, neural precursor cell lines (Bartlett et al., 1988; Frederickson et al., 1988; Snyder et al., 1992, Pietsch et al., 1994). However, transformation frequently results in alteration of the neural precursor cell properties and instability of the differentiated phenotype of the cells; for example, SV40 large T-antigen transfection of precursor cells subverted the establishment of cerebellar granule cell identity (Gao and Hatten, 1994). Additionally, v-myc transfected multipotent mouse cerebellar derived cell lines can spontaneously alternate between neuronal and glial phenotypes (Snyder et al., 1992). CNS germinal cells immortalized by *v-myc* appear "plumper" than controls when transplanted into cerebellum. Because of their "immortalized" phenotype, these cells are not ideal for cell implantation therapies.

There is increasing evidence that the hormonal and neurotrophic factor requirements and responses of neural cells change as they progress through development (Cattaneo and McKay, 1990; Verdi and Anderson, 1994; Nurcombe et al., 1993; Segal, et al., 1992; Gao et al., 1992; Gao et al., 1995). Recent evidence suggests that it might be possible to halt neuroepithelial cell differentiation and allow for indefinite proliferation of murine neural precursor cells in serum-free medium supplemented with growth factors appropriate for the desired cell type at the desired stage of differentiation. A glial precursor cell line (SFME), dependent on EGF, was established in serum-free medium with defined supplements (Loo et al., 1987). EGF was also found to support the indefinite growth of subventricular neural progenitor cells (Reynolds and Weiss, 1992) and retinal neural precursor cells (Anchan et al., 1991). Meanwhile, FGF has been found to be a critical factor for the survival, proliferation, and differentiation of certain CNS precursors and neural crest cells (Frederickson et al., 1988; Murphy et al., 1990; Cattaneo and McKay, 1990, Kilpatrick

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Author to whom all correspondence and reprint requests should be addressed: Jennie P. Mather, Ph.D., Genentech, 460 Point San Bruno Blvd., South San Francisco, CA 94080. E-mail: mather.jennieNgene.com

and Bartlett, 1993; Murphy et al., 1994; Vicario-Abejon et al., 1995; Ghosh and Greenberg, 1995). However, neither EGF nor FGF alone, or in combination with serum, support the long-term survival of neuroepithelial cells cultured from earlier stages of development (e.g., E9 rat neural plate). Given their developmental potential and the importance of these cells as precursors of later stages of neural differentiation, we attempted to grow neuroepithelial cells from E9, 0-somite rat embryos in serum-free hormone-supplemented medium. Here we report the isolation, establishment and characterization of an early neuroepithelial precursor cell line that can be induced to differentiate into a variety of neural cell types in vitro and in vivo.

Results

Neuroepithelial Cell Growth

Neuroepithelial cells from dissected neural plates from E9 (0-somite) rat embryos survived poorly in most culture conditions. No cells survived beyond 4 d in conditions supplemented with EGF, FGFs, IGFs, and neurotrophins alone or in combination (Fig. 1A). However, significant cell survival and proliferation was found in primary neural plates when cocultured with an embryonic Schwann cell line (nrESC, Li et al., 1996a) monolayer in the presence of the optimal Schwann cell growth medium which contained insulin (ins), transferrin (Tf), α-tocopherol (α-T), progesterone (prog), forskolin (for), bovine pituitary extract (BPE), and recombinant human heregulin (rh-HRG) (Li et al., 1996a). Under these conditions, approximately 10% of the originaily plated epithelial cells rapidly proliferated to form large cell colonies that pushed away the Schwann cells or grew underneath the Schwann cell monolayer (Fig. 1B). These cell colonies formed secondary colonies after subculture (Fig. 1C). Addition of bFGF and retinoic acid to the secondary cultures induced the cells to extend long processes and assume a morphology resembling that of neurons (Fig. 1D).

In order to avoid the coculture step and more readily achieve a neuroepithelial monoculture, we tested embryonic Schwann cell conditioned medium (ESCCM) for its ability to support the growth and survival of this cell type from E9 neural plate cultures In addition, we set up cultures with trans-well inserts containing Schwann cells. The insert separates the Schwann cells from the neuroepithlial cells by a physical barrier but allows many Schwann cell-secreted molecules to pass through the membrane. Both methods resulted in the survival and proliferation of a small proportion of neuroepithelial cells, which proliferated to form large epithelial colonies containing a few differentiated neurons after 10 d in vitro. No viable cells were detected in conditions without ESCCM or coculture. The cells grown in the presence of ESCCM were successfully subcultured. During the first five to six passages, the cultures comprised a mixture of cell types derived from the E9 neural plate, the

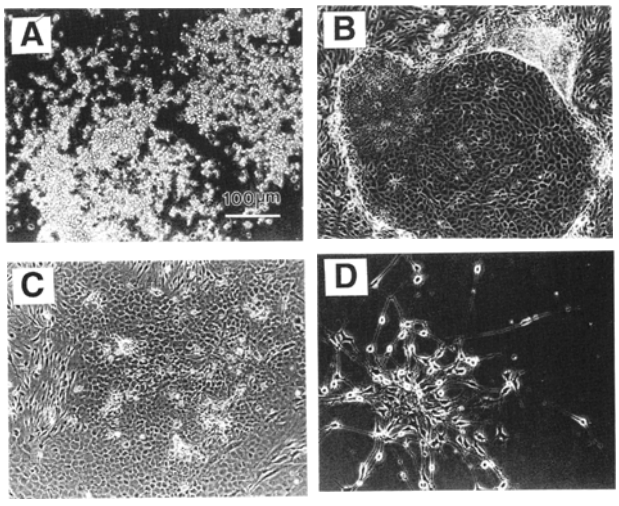

Fig. 1. Cultures of E9 neural plate cells. **(A)** Debris of dead cells (confirmed by trypan blue exclusion test) in cultures grown in 7F (insulin, Tf, BPE, forskolin, hrg, prog, αT) **(B)** Primary cell colonies formed in 7F in coculture with the Schwann cell line, ESC, in 7 d. **(C)** Secondary colonies formed after subculture (passage 1, day 5). **(D)** The cells, cultured in the presence of 7F + bFGF and Vitamin A showed a marked change in morphology. Magnification is indicated by the bar on the figure, all panels were taken at the same magnification.

epithelial-type cells, spindle-shaped cells resembling Schwann cells or radial glia, and aggregated small cells of a neural morphology with long, fine processes. Secondary cultures no longer required the Schwann cell-conditioned medium for growth. The epithelial type of cells were enriched by differential enzymatic digestion at subculture (nonepithelial cells detached from the plate first and were discarded) by removing heregulin (a potent glial cell mitogen) from the culture medium, and by keeping the cells growing at high density. Under these conditions the epithelial cells became the predominant cell type within a few subcultures. This cell type has formed a cell line, designated NEP (neuroepithelial progenitor), capable of extended passage in vitro.

In our experiments, the initiation of cultures required that the primary cells not be dissociated and, in addition, required the presence of soluble factor(s) from Schwann cells, because the NEP-like cells survived and proliferated only in the coculture with embryonic Schwann cells or in the presence of freshly prepared ESCCM. Although the NEP line did not require ESCCM when carried in high density culture, coculture with Schwann cells enhanced the survival and proliferation of NEP aggregates in low-density culture (data not shown). This effect of ESCCM could not be substituted for by any of the known growth factors or neurotrophins tested. The identity of the activity in the ESCCM remains to be elucidated.

These epithelial cells, and the NEP cell line, required cell–cell contact for survival and growth. Dispersion of the culture to single cells, even with a brief trypsin treatment,

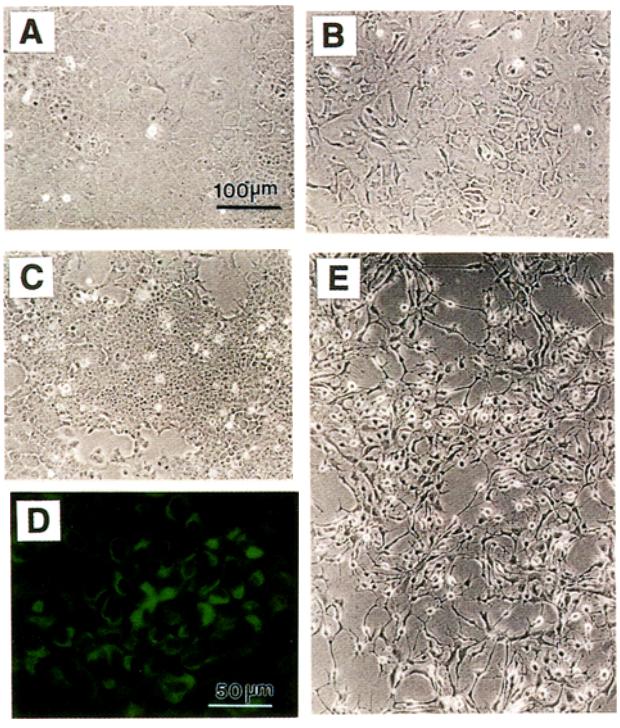

Fig. 2. In vitro differentiation of NEP cells. The NEP cell line at passage 35 was grown with insulin, TF, prog, αT, and BPE (5F) **(A)**, with these 5F plus bFGF (30 ng/mL) **(B)**, 5F plus forskolin (5 μ*M*) **(C)**, or with 5F, bFGF, and forskolin **(E)** for 48 h. The morphology of NEP cells grown for 50 population doublings in the presence of 6F and immunostained for nestin is shown in **D**. Magnification is indicated by the bar on the figures, A–C and E were taken at the same magnification.

resulted in cell death even when the cells were replated at a very high plating density. Single cells resulting from collagenase-dispase treatment did not survive when plated, only those cells that attached in small aggregates survived. Optimal growth was achieved when cell clumps (5–20 cells) were plated at a density of 2–3 × 10^5 cells/cm^2. A population doubling time of 50 h was calculated for the NEP line. Confluent cultures reached a density of 10^6 cells/cm^2 but always remained a monolayer. This cell line was not cloned due to its inability to survive as single cells. To date, the cells have been grown continuously for over 40 passages (>80 population doublings), no obvious change has been found in cell morphology nor the growth profile or hormone response and, no cell senescence has been observed.

NEP Cells Are Nestin Positive

The cell line showed a monolayer epithelial cell morphology with a large nucleus/cytoplasm ratio during routine subculture (Fig. 2A,C). All cells showed positive immunofluorescent staining for the neural precursor cell-specific intermediate filament protein, nestin (Fig. 2D). A western blot of solubilized cell membranes stained with antinestin antibody showed a single band at mol wt 220 kDa, consistent with reported mol wt of nestin (data not shown). Cells maintained positive staining for nestin even when treated with bFGF and forskolin. A small proportion (<3%) of cells, located near the periphery of colonies, also showed positive staining for neuron-specific enolase (NSE) (*see* Table 1) which had the expected mol wt of 46 kDa on a western blot. These NSE-positive cells may represent a small proportion of the cells that continuously differentiate along the postmitotic neuronal pathway and are lost from the culture at each passage.

Growth Factor and Attachment Factor Response

The NEP cell line required the presence of insulin, BPE, and forskolin (or cholera toxin or cAMP analogs) for survival and growth. Removal of any one of these three growth factors resulted in a sharp decrease in cell number (Fig. 3A). The BPE could not be substituted for by any of the commercially available hormones or growth factors known to be present in the pituitary (Table 2). Unlike many neural-derived cell lines (Bottenstein, 1985), these cells showed no growth response to Tf when grown in the presence of soluble iron salts. Optimal concentrations of these growth factors were determined to be 10 μg/mL insulin, 3–10 μ*M* forskolin, and 0.3% (v/v) BPE (Fig. 3B,C). Insulin could be replaced by insulin-like growth factors. The cells were more sensitive to IGF-1 than to IGF-II (Fig. 3D). Growth factors such as PDGF, EGF, leukocyte inhibitory factor (LIF), hepatocyte growth factor (HGF) and neurotrophins (NGF, NT-3 and BDNF) (Fig. 3E,F), and heregulin (hrg) (Fig. 3G) did not increase cell number. Members of the TGFβ family of growth factors had an inhibitory effect on cell growth with addition of TGFβ1 resulting in significant cell death (Fig. 3G). The NEP cells attached and grew only on laminin-coated plates. Cells plated on plastic or plastic coated with poly-D-lysine, fibronectin, or collagen failed to attach and died within 12 h. Cells plated on EHS matrix attached and survived but did not grow (data not shown). All factors tested and their effects on NEP cell growth are summarized in Table 2.

Basic fibroblast growth factor (bFGF) had a marked growth inhibitory effect on the cells (Fig. 3H). Additionally, while all the cells died in F12/DME supplemented with insulin only, with the addition of bFGF, a large proportion of cells survived as floating cell aggregates (data not shown). Addition of bFGF in the presence of forskolin and the other growth factors greatly inhibited cell proliferation, completely blocking cell growth beyond the first 24 h of treatment (time lapse video, data not shown). In contrast, aFGF showed little effect (Fig. 3H).

In Vitro Differentiation of the NEP Cell Line

The cells cultured with bFGF, in the presence of the other growth factors, remained viable but ceased to grow and underwent a marked change in morphology, becoming less epithelial in appearance (Fig. 2A,B). Forskolin, without bFGF, increases cell division but does not cause a change in morphology (Fig. 2C). However, in the presence

Table 1
Induction of Neuronal Markers in NEP Cells In Vitro by bFGF and bFGF With Forskolin[a]

Antibodies	Control	bFGF	Forskolin +bFGF
General neuron markers			
Neuron specific Enolase	Rare	30–50%	>90%
Tubulin-beta	Rare	>90%	>90%
Microtubule associated protein-2	10–20%	>90%	>90%
Neurofilament 68 kDa	Negative	10–20%	10–20%
Neurofilament 160 kDa	Rare	Weak	Strong > 90%
Neurofilament 200 kDa	Negative	Negative	3–5%
Synaptophysin	Negative	Rare	>90%
Tau	Negative	Negative	50%
Cortical neuron marker			
Protein kinase C-γ (1.12)	Rare	>90%	>90%
Neurotransmitters and synthesizing enzymes			
Glutamate	Weak	Weak	Strong >90%
Aspartate	Weak	Weak	Strong >90%
Serotonin	Negative	Negative	Negative
Choline acetyl-transferase	Negative	3–5%	3–5%
Tyrosine hydroxylase	Negative	Negative	Negative
Oligodendricyte markers			
Gal C	Negative	Negative	Negative
HNK-1	Negative	Negative	Negative
Astrocyte marker			
GFAP	Negative	Negative	Very rare
Peripheral nerve			
Peripherin	Negative	Rare	3–5%
General neural markers			
P75-LNGFR	10–20%	10–20%	10–20%
Vimentin	Rare	>90%	>90%

[a]NEP cells were cultured in control (6F) medium with the addition of bFGF or bFGF and forskolin for 120 h. For immunocytochemistry, cells were fixed and stained, with the antibodies and concentrations listed in Table 3. The percentage of positively stained cells was determined by counting the cells in three randomly selected fields ("rare" is <3% positive cells). Positive cells were counted under bright field microscopy and total cells were counted under phase contrast. Total cell counts exceeded 500 cells/sample. The results of three separate experiments were consistent and the data were presented as the range of results of all three experiments.

of forskolin, the differentiation seen with bFGF was more marked. The cells had rounded cell bodies with multiple cell processes, some extending for a considerable distance. These cells appeared quite neuronal in phenotype (Fig. 2E). This cessation of cell growth coupled with the distinctive change in morphology suggested that the cells might be differentiating further down a neuronal pathway.

To test this hypothesis, cells were grown in the presence of 5F alone (insulin, TF, BPE, progesterone, α-tocopherol), 5F + bFGF, or 5F + bFGF + forskolin, for 96 h and then fixed and immunostained for a variety of markers associated with neurons (such as neurofilament filament proteins NF 68, NF 160, NF 200, tau and synaptophysin, NSE, and MAP2), oligodendrocytes (GalC, HNK-1), and astrocytes (GFAP). To further differentiate between classes of neurons we stained for neurotransmitters (e.g., glutamate, aspartate, serotonin), the cortical neuron marker PkC (1.12) and the peripheral nerve marker, peripherin. The staining is

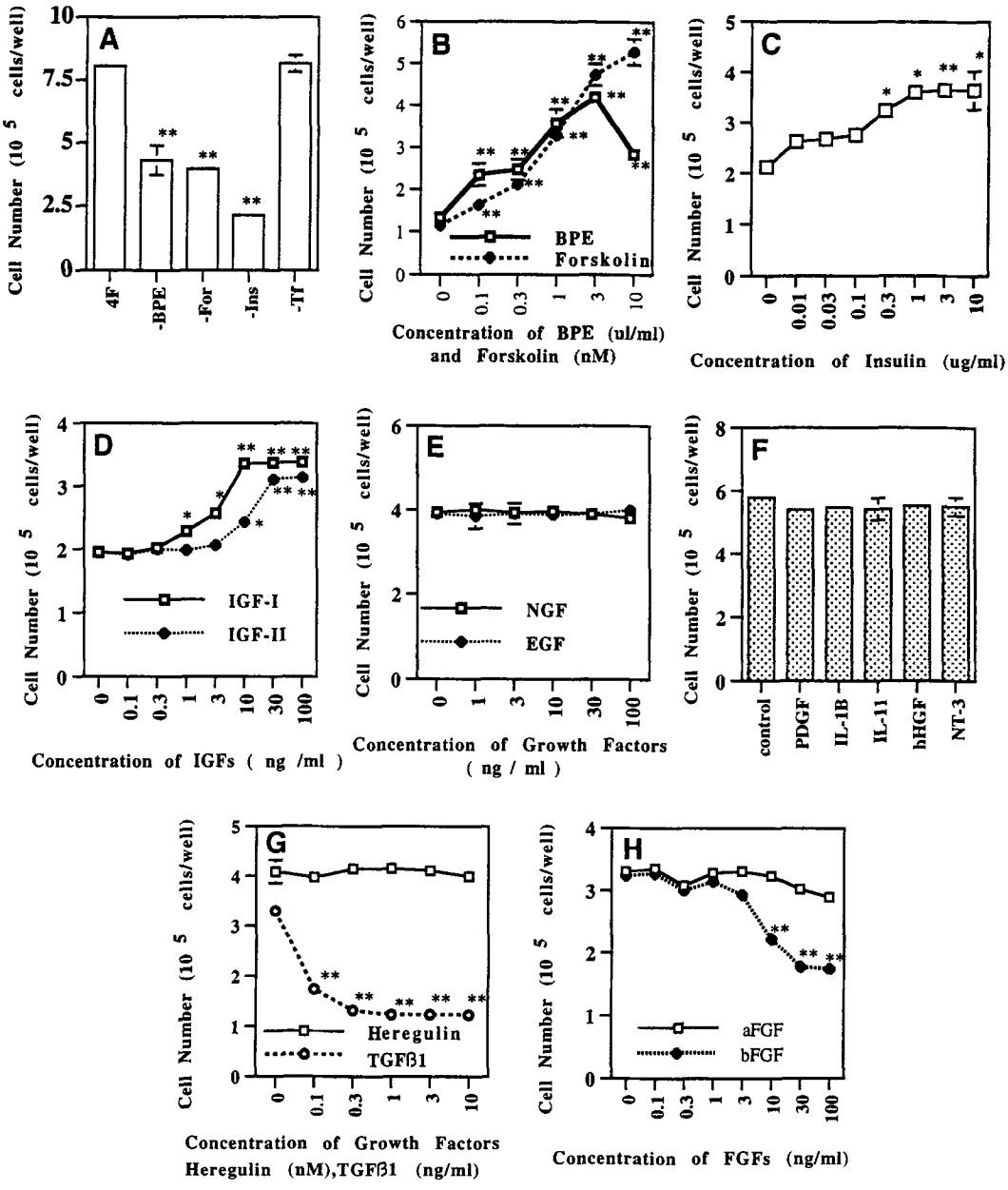

Fig. 3. NEP cell growth response in vitro. Cells were grown as described omitting only the factor indicated **(A)**. In A, "4F" indicates all factors (BPE, For, Ins, and Tf) were added. In panels **B–D**, cultures were exposed to the indicated concentrations of forskolin, BPE (B), or insulin (C) in the presence of all other factors in 6F (*see* Fig. 1). The response to IGF-I and IGF-II is shown in the absence of insulin (D). The response to NGF **(E)**, EGF (E), heregulin **(G)**, TGF-β1 (G), FGF's **(H)**, and other growth factors **(F)** (PDGF,10 ng/mL; IL-1β, 5 ng/mL; IL-11, 5 ng/mL; HGF, 300 ng/mL; NT3 10 ng/mL) is shown in the presence of the complete 6F. Values shown are the mean ± sem of cell counts of four replicate cultures. Error bars not visible are smaller than the symbol. Statistical analysis was performed using the student's *t*-test: $*p < 0.05$, $**p < 0.01$.

summarized in Table 1. The morphological changes seen in the presence of bFGF, and even more in cells grown in bFGF and forskolin, were accompanied by the increased expression of neuronal specific markers visualized by immunohistochemistry.

Staining for two intermediate filament proteins, tubulin β and vimentin is shown in Fig. 4. The number of cells expressing, and the intensity of staining for both vimentin (Fig. 4A,C,E) and tubulin β (Fig. 4B,D,F) were increased by bFGF (Fig. 4C,D) and further increased by the combination of bFGF and forskolin (Fig. 4E,F). Specific neuronal markers, such as MAP-2 (Fig. 5A,D,G), the 160-KDa neurofilament protein (NF 160) (Fig. 5 B,E,H), and peripherin (Fig. 5C,F,I), were also induced as the cells became more differentiated morphologically although these were expressed to differing extents and in various ways under the different conditions. For example NF 160 was expressed only in a few cell–cell junctions in the control conditions (Fig. 5B). With bFGF alone, there was some cytoplasmic expression (Fig. 5E) while in the bFGF +

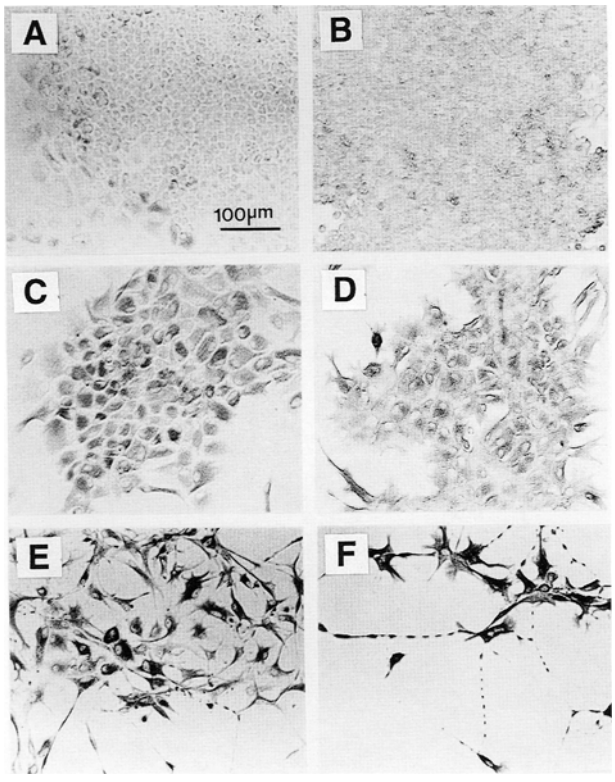

Fig. 4. NEP cells acquire neural markers in vitro. NEP cells were grown in the presence of 5F alone (ins, TF, BPE, prog, vit E) **(upper panel)**; 5F + bFGF (30 ng/mL) **(middle panel)**; or 5F + bFGF + forskolin (5 μ*M*) (lower panel) for 96 h and then fixed and immunostained for the intermediate filaments vimentin **(A,C,E)** or tubulinβ **(B,D,F)**. Magnification is indicated by the bar on the figure, all panels were taken at the same magnification.

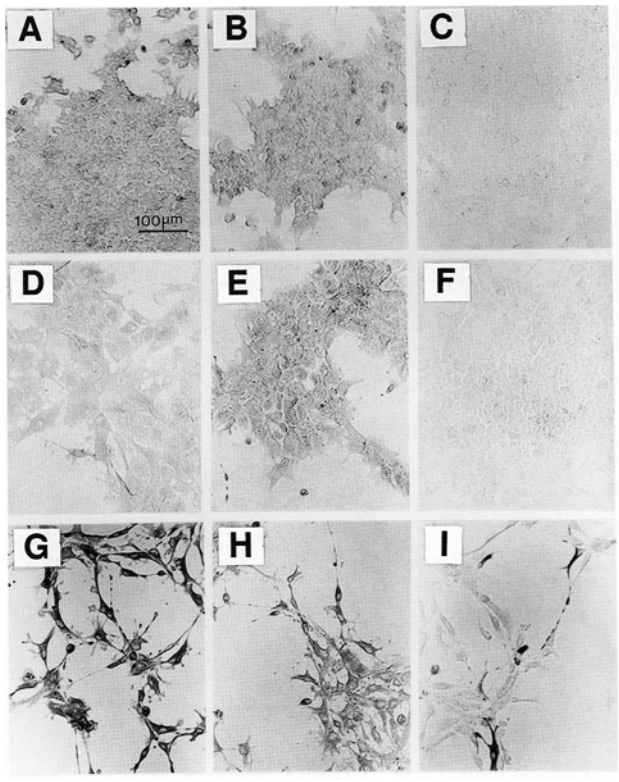

Fig. 5. NEP cells acquire neural markers in vitro. NEP cells were grown in the presence of 5F alone **(A,B,C)**; 5F + bFGF (30 ng/mL) **(D,E,F)**; or 5F + bFGF + forskolin (5 μ*M*) **(G,H,I)** for 96 h and then fixed and immunostained for the neuronal markers MAP 2 (A,D,G), neurofilament 160 (B,E,H), and peripherin (C,F,I). Magnification is indicated by the bar on the figure, all panels were taken at the same magnification.

forskolin conditions NF 160 could be seen in cytoplasm of the cell body and the neural extensions (Fig. 5F). While MAP-2 was expressed by most cells in the presence of both FGF and forskolin (Fig. 5G), peripherin was present on only a few of the cells (Fig. 5I). Cells exposed to both factors expressed protein kinase C (PKC) (Fig. 6A,D,G), as well as the excitatory amino acids glutamate (Fig. 6 B,E,H) and aspartate (Fig. 6C,F,I). The cells were negative for staining for serotonin and tyrosine hydroxylase. A small proportion (3–5%) of cells showed staining for choline acetyl-transferase when bFGF was present (Table 1) Neuron-specific enolase, synaptophysin, and Tau were also strongly induced in the cultures exposed to both bFGF and forskolin. The p75 NGF-R was present in low levels in the cultures and not affected by bFGF or forskolin. Glial markers such as GFAP and Gal-C and HNK-1 were not present in any of the conditions tested in vitro (Table 1).

In Vivo Differentiation of NEP Cells

In order to determine the extent of the potential for differentiation of the NEP cell line, we injected labeled NEP cells into neonatal rat brain. This allowed us to determine whether NEP cells were capable of integration into the brain, and what types of cells it could generate in different in vivo environments. When cells labeled with the fluorescent viable cell marker, PKH-26 (Gao and Hatten, 1993) were injected into neonatal rat brain, the NEP cells integrated and gave rise to several distinct cell phenotypes. Integrated cells had a morphology characteristic of granule cells and Bergmann glia (Fig. 7A,B) within the appropriate layer of the cerebellum (Gao and Hatten, 1994). In the hippocampus, most of the fluorescently labeled cells integrated into the dentate gyrus. The majority of these cells had a morphology typical of dentate granule cells with the cell bodies located in the granule cell layer and dendritic projections to the molecular layer (Fig. 7C,D). Other cells showed neuronal-like differentiation but could not be precisely categorized by morphology. In the cerebral cortex, bundles of cell processes spanned the region from the ventricular layer up to the surface of the cortex, with terminal branches that resembled radial glial cell processes (Fig. 7E) (Rakic, 1972; Fishell, 1995). Some of the cell bodies were traced and were located on the lateral ventricular lining.

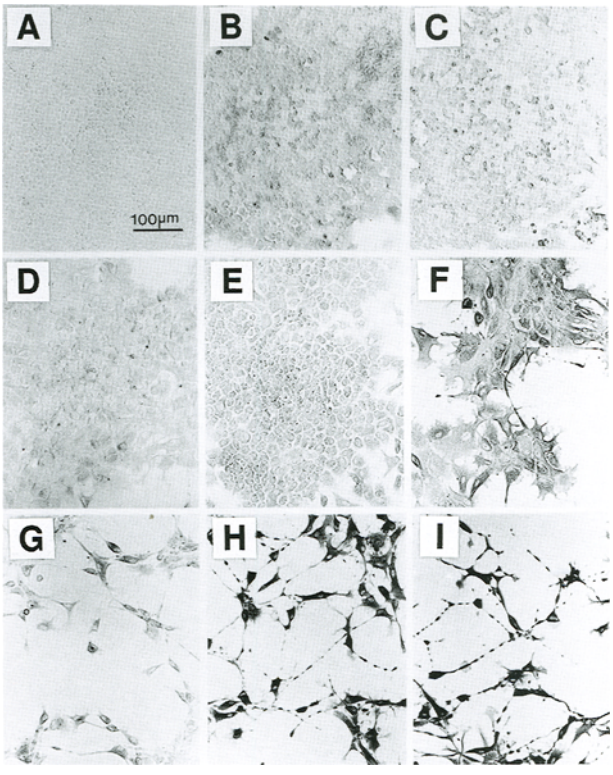

Fig. 6. NEP cells acquire neural markers in vitro. NEP cells were grown in the presence of 5F alone **(A,B,C)**; 5F + bFGF **(D,E,F)**; or 5F + bFGF + forskolin **(G,H,I)** for 96 h and then fixed and immunostained for the neuronal markers neuron-specific PKC isozyme γ (A,D,G), and the neurotransmitters glutamate (B,E,H), and aspartate (C,F,I). Magnification is indicated by the bar on the figure, all panels were taken at the same magnification.

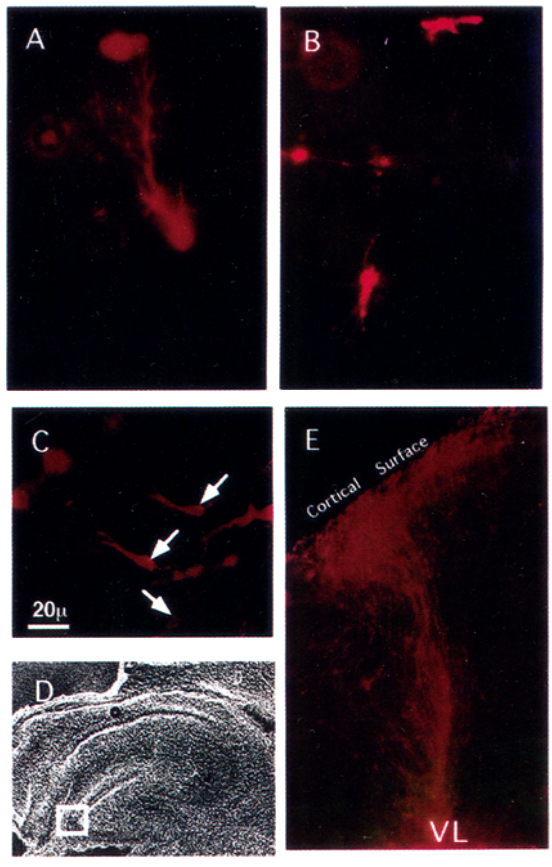

Fig. 7. In vivo differentiation of NEP cells labeled with PKH-26 and implanted into P1 rat brain. In the cerebellum, PKH-labeled cells developed mophologies typical of granule neurons (small neuron with T-shape ascending processes) **(A)** or Bergmann glial cells **(B)**. Panels A and B show photographs from cerebellar coronal sections fixed 4 d after implantation (EGL, external germinal layer; ML, molecule layer; PCL, purkinje cell layer; IGL, internal granule layer). In the hippocampus, PKH-labeled cells in the granule cell layer of the dentate gyrus developed a granule cell morphology **(C,D)**. Panel C is a magnified fluorescent photograph of the boxed area of the section shown in *D*. Panel D shows a phase contrast microphotograph of a longitudinal section of the hippocampus (fixed 5 d after implantation). In the cerebral cortex, long PKH-labeled radial glial cell processes spanned the area from the lateral ventricle lining **(VL)** to the surface of the cortex **(E)**, (fixed 5 d after implantation). Magnifications are indicated on the photographs.

Discussion

We have derived culture conditions that selectively support the survival and growth of a subset of cells present in the early neural plate. Cells with the properties described can be cultured from embryos of the 0-somite stage of development (day 9) only during a 3-h time period. NEP cells, derived from E9 neural plate, have properties substantially different from those of cell lines derived from more differentiated rat (E13-P1) and mouse (E10-P1) brain. When carried under the conditions described, NEP cells remain arrested in this precursor stage and continue to divide and express high levels of nestin (Hockfield and McKay, 1985; Lendal et al., 1990, Loo et al, 1994) and low levels, or none, of markers for differentiated neurons.

The NEP cells are characterized by a requirement for a laminin substrate, one of the first extracellular matrix proteins expressed in embryonic development. In addition, cell–cell contact, seemingly mediated by a cell surface protein, is required for survival. They have mitogenic responses to insulin (or IGFs), forskolin, and BPE, but not FGF, EGF, or neurotrophins, as described for previously established cell lines (Loo, et al., 1987; Bartlett et al., 1988; Frederickson et al., 1988; Snyder et al., 1992; Pietsch et al., 1994).

Both bFGF and forskolin are required for effective neuronal differentiation. When exposed to these two factors, the NEP cells ceased active cell division and >90% of the cells expressed of neuronal markers. This very high level of differentiation, not previously observed in other cell lines, might be because of growing the NEP cells in defined conditions devoid of differentiation factors allowing the cells to remain responsive to differentiating signals. Finally, the cells can integrate into the developing brain and give rise to appropriate postmitotic neurons in vivo, with properties distinct from those of other precursor cells reported in the literature.

Table 2
Summary of Growth Factors Tested for the Growth of Neuroepithelial Cells

Growth factors	Primary culture	NEP
Insulin (ins)	+	+
Insulin-like growth Factor I (IGF-I)	+	+
Insulin-like growth factor II (IGF-II)	+	+
Epidermal growth factor	0	0
Heregulin (hrg)	+	0
Platelet-derived growth factor (PDGF)	0	0
Hepatocyte growth factor (HGF)	0	0
Growth arrest-specific protein 6 (Gas6)	0	0
TGF-β-1,2,5	–	–
hr-Activin A	–	–
hr-Inhibin A	0	0
Human recombinant (hr) Follistatin	0	0
Fibroblast growth factor, acidic (aFGF)	–	–
Fibroblast growth factor, basic (bFGF)	–	–
Nerve growth factor (NGF)	0	0
Neurotropin-3 (NT3)	0	0
Neurotropin-4/5	0	0
Brain-derived growth factor (BDNF)	0	0
Ciliary neurotrophic factor	0	0
Interleukin-1α	0	0
Interleukin-1β (IL-1β)	0	0
Interleukin-11 (IL-11)	0	0
Leukemia-inhibiting factor (LIF)	0	0
Stem cell factor (SCF)	0	0
Colony-stimulating factor (CSF)	0	0
Forskolin	+	+
BPE	+	+
Follicle-stimulating hormone (FSH)	0	0
Luteinizing hormone (LH)	0	0
Growth hormone (rhGH)	0	0
Adrenal cortical tropic hormone (ACTH)	0	0
Gonadotropin-releasing hormone (GnRH)	0	0
Calcitonin gene-related peptide	ND	0
Vasoactive intestinal peptide (VIP)	ND	0
Thyroid-stimulating hormone (TSH)	ND	0
Thyroxine T3	0	0
Thyroid hormone-stimulating hormone-releasing hormone (THRH)	ND	0
Somatostatin	0	0
Laminin	+	+
Collagen I	–	–
Fibronectin	0	0
Poly-D-lysine	0	0

[a] +, 0, –: Scored at level of $p = 0.05$ by Student t-test; +: growth stimulatory. – : growth inhibitory. 0: no growth response. ND: not determined. Hormones and growth factors were tested at different concentrations through a range of at least two orders of magnitude arround the respective ED50 suggested by suppliers in F12/DME medium in the presence of Tf, α-T, and prog and with/without insulin, forskolin and BPE. For primary cultures the factors were tested on cocultures of the ESC Schwann cell line.

Growth of Neural Precursor Cells

The adult mammalian brain contains several distinct cell types. Most of these cells, including neurons, astrocytes, and oligodendrocytes, are derived from neural precursors during development. The fates of these precursor cells are largely determined by cell–cell and cell–substrate, including the presence or absence of the correct growth factors and extracellular matrix proteins. Basic FGF is a potent mitogen for certain CNS populations (Murphy et al., 1990; Cattaneo and McKay, 1990; Kilpatrick and Bartlet, 1993; Ray and Gage, 1994; Ray et al., 1993; DeHamer et al., 1994; Ghosh and Greenberg, 1995; Vicarrio-Abejon et al., 1995), while bFGF and NGF cooperatively promote the proliferation and differentiation of embryonic striatal precursors (Cattaneo and McKay, 1990). EGF influences the proliferation of retinal neuroepithelial cells (Anchan et al., 1991), supports the survival of neural precursor cells from adult mouse striatum (Reynolds and Weiss, 1992) and is required for the survival of SFME, a glial precursor cell line (Loo et al., 1987). Neither FGFs nor EGFs stimulated the growth of the NEP cells.

In contrast, growth factors of the insulin/IGF family are some of the most critical components of the growth medium for NEP cells. This is consistent with reported mitogenic activity of IGFs on cultured rat sympathetic neuroblasts (DiCicco-Bloom and Black, 1988) and mouse cerebellar EGL cells (Gao et al., 1991). Additional factors, such as the activity in pituitary extract, are essential for NEP survival and proliferation. Forskolin may substitute in vitro for cAMPmediated factors which stimulate the proliferation of neuroepithelial precursor cells in vivo.

The growth and survival of NEP cells requires cell–cell contact. This contrasts with the phenotype of all other established neural precursor cell lines, but is consistent with results from primary embryonic cultures. In these primary cultures, only cells in clusters survived, proliferated and remained nestin-positive (Cattaneo and McKay, 1990; Ghosh and Greenberg, 1995; Vicarrio-Abejon et al., 1995). Additionally, cell aggregation stimulated the proliferation of cerebellar neuroblasts (Gao et al., 1991) and retinal neuroepithelial cells (Watanabe and Raff, 1990). In the developing brain, only those stem cells in the ventricular zone continued to divide and these cells became differentiated when they migrated away from this region. Davis and Temple (1994) described membrane associated factors in sonicated C6 glioma cell membrane homogenates which maintained the cell division of isolated cortical progenitor cells. It was suggested by Ghosh and Greenberg (1995) that a membrane-bound factor might mediate the mitogenic effect of bFGF in their cultures. There is a growing body of evidence that cell–cell contacts can be mediated by receptor/ligand-like pairs of molecules, for example sevenless and bride of sevenless (Kramer et al., 1991), which play crucial roles in regulating development. The absolute requirement for cell–cell contact for the survival and continued growth of NEP cells may reflect the role of such pairs of molecules in this early stage of neural development and the later developing subventricular zone.

Induction of Neuronal Differentiation

Within 24 h of exposure to bFGF the cells withdrew from the cell cycle and became less dependent on mitogenic factors for survival. Thus, bFGF rapidly altered both the mitogenic state of the cells and their hormone responsiveness. Forskolin seemed to increase the rate and/or extent of bFGF-induced differentiation of NEP cells. This response to bFGF is in marked contrast to that reported for existing cell lines and primary cultures of many types of cells. For some neural precursor cell cultures, the removal of mitogenic bFGF is a prerequisite for the onset of neuronal differentiation (Cattaneo and McKay, 1990; Kilpatrick and Bartlet, 1993). In contrast, Vicarrio-Abejon et al. (1995) found that bFGF or NT-3 induced the differentiation of calbindin-positive cells in primary cultures of hippocampal cells. In NEP, maximal neuronal differentiation required the synergistic action of bFGF and a protein kinase A activator. This requirement for elevated cAMP is consistent with the induction of neuronal differentiation in embryonic carcinoma cells (Eddé and Darmon, 1985), embryonic stem cells (Fraichard et al., 1995) and certain immortalized neural precursor cells (Frederickson et al., 1988). However, retinoic acid is not a prerequisite for NEP cell differentiation as it is for those undifferentiated embryonic cells (Eddé and Darmon, 1985). In addition, the fact that NEP cells retain nestin immunostaining (Loo et al., 1994) and fail to express any glial cell markers in vitro suggests that they are not differentiating down the glial pathway.

The majority of the neurons differentiated from NEP in vitro in the presence of bFGF and forskolin displayed markers consistent with a cortical neuron identity. The differentiated neurons expressed a neuron-specific PKC isoenzyme-γ that is expressed exclusively in CNS cortical neurons and accumulated the excitatory amino acids, glutamate and aspartate, in their cytoplasm and in cell processes. This in vitro data is confirmed and strengthened by the results of the in vivo experiments. When implanted into neonatal rat brain, NEP cells survived, integrated, and differentiated into the neuronal cell types which normally express this PKC isozyme and use glutamate and aspartate as transmitters. However, in these experiments, the NEP cells were pretreated with bFGF before implantation which might have lead to some restriction of their subsequent developmental potential in vivo.

Although no mature glial cells were identified in vitro, the NEP cells can differentiate into cells morphologically resembling Bergmann glial cells if injected into the cerebellum; and radial glial cells (Hunter and Hatten, 1995) if injected into the cerebral cortex. As yet unknown endogenous factors or components of the local microenviron-

ment may be required for the NEP cells to differentiate into glial cells. In addition, the bFGF pretreatment before injection may have limited the number of cells capable of differentiating along these pathways. Further experiments are in progress to determine what factors predispose the NEP cells to differentiate along other glial or neuronal pathways.

These data suggest that the NEP cell line is arrested at an early stage of neuroepithelial differentiation and maintains the ability to differentiate into a wide variety of neuronal and glial cells depending on the environment This is in agreement with observations by other investigators using more differentiated cells. In the PNS, whether a bipotential neural crest cell differentiates to a sympathetic neuron or to a Chromaffin cell is determined by environmental NGF and glucocorticoids (Anderson and Axel, 1986; Vogel and Weston, 1990). Glial growth factor restricts neural crest stem cells to a glial fate (Shah et al., 1994). In the CNS, multipotential precursor cells differentiate into different neurons and glial cells according regional and positional cues. Floor plate and notochord, or sonic hedgehog produced in these tissues, induced nearby neural precursor cells to adopt a motor neuron (Roelink et al., 1994; Roelink et al., 1995) or dopaminergic neuron fate (Hynes et al., 1995a, b). Implantation of neural precursor cells from one region of the brain can change their fate to that of the neighboring cells of the host brain (Fischell, 1995). Therefore, because of the early stage of differentiation represented by the NEP cells, it should be possible to drive NEP cells down a number of different developmental pathways by manipulating the in vitro or in vivo environment. It is hoped such studies will expand our understanding of the developmental regulation of the neuroepithelial precursor cells from this early stage of mammalian development.

Materials and Methods

Animals

Pregnant Sprague-Dawley rats were obtained from Charles River (Hollister, CA) and kept in a controlled temperature and humidity enviroment, with a light period of 0600–1800 h food and water were supplied ad libitum. The animals were sacrificed on day 9 of pregnancy and E9 embryos were collected.

Primary Culture

E9 rat neural plates (0-somite) were dissected under a dissecting microscope using fine needles. The caudal portion was removed by cutting through the middle of Hensen's node. Mesoderm and endoderm were removed after a brief incubation with collagenase and dispase (Boehringer Mannheim, Mannheim, Germany 0.2%,10 min at 0°C). The clean neural plates were washed 5× by transfer from plate to plate of F12/DMEM with 1% SBA. The neural plates were then dispersed into small aggregates (preferentially 20–50 cells in each aggregate) by gentle pipeting with a 200 Pipetman pipet. Complete dispersion must be avoided. The dispersed cells were plated in 96-well microtiter plates (cells from 20 neural plates were divided into 96 wells) precoated with attachment factors (*see* Li et al., 1996a,b) in serum-free medium supplemented with different combinations of growth factors. Good cell survival and growth was found only on laminin-coated plates in medium which contained BPE (Roberts et al., 1989), insulin, and forskolin, and additionally were either cocultured with the rat embryonic Schwann cell line (ESC, Li et al., 1996b) or with 10-fold concentrated ESCCM. In these conditions, some precursor cells survived and proliferated to form large colonies of compacted monolayer epithelial cells containing some differentiated neurons bearing long processes. Cells in other conditions died within 4 d in culture.

For transwell coculture, cell culture inserts (Falcon Cat #3090) with transparent Cyclopore membrane 0.45-μm pore size were coated with laminin. Schwann cells were plated inside the inserts for at least 24 h before using. Then the inserts were transfered into laminin-coated 6-well plates which were freshly plated with neural tube cells.

Long-Term Culture

Colonies of epithelial cells formed in the primary culture in F12/DMEM (Gibco-BRL, Grand Island, NY) supplemented with 7F (insulin, 10 μg/mL; transferrin, 10 μg/mL; progesterone, $3 \times 10^{-9}M$; α-tocopherol, 5 μg/mL; BPE, optimal concentration for growth stimulation determined for each batch prepared as described in Roberts et al., (1990), 3 μL/mL); forskolin, 5 μ*M*; recombinant human heregulin (Genentech, Inc., South San Francisco, CA, HRG-β1 177–244, 10 n*M*) and ESCCM, were removed from the substrate with 0.2% collagenase/dispase (Boehringer- Mannheim) at 37°C. The cells were washed free of the enzymes by centrifugation on a layer of 3% BSA and plated onto laminin-coated 24-well plates with F12/DMEM supplemented with 7F. The cells were routinely subcultured by the same procedure for 5–6 passages. During this period the cultures contained two major cell types, one is the compact epithelial cells, the other is bipolar resembling Schwann cells or radial glia. These cells were subsequently removed from the culture by differential enzymatic digestion with collagenase dispase (Boehringer Mannheim), (these cells lifted off the plate first and were discarded), removing heregulin from the culture medium, and allowing the epithelial cells to grow at a higher density. The cells were then carried in 6F medium (7F medium without heregulin) on laminin-coated plates and passaged every week with collagenase dispase, using a 1:4 split. Hormones, attachment factors, and growth factors were obtained from one of the following sources: Calbiochem, La Jolla, CA; Sigma, St. Louis, MO; Novo Nordsk, Denmark; Gibco-BRL; or Upstate Biotech, Lake Placid, NY. The cell line has been deposited with the American Type Culture Collection (ATCC).

Cell Proliferation Studies

Cells between passages 25 and 50 (50–100 population doublings) were used in the experiments. Cells were routinely detached from the culture vessels using collagenase-dispase digestion and plated at the density of 2×10^5 cells per well on laminin-coated 24-well plates in F12/DMEM medium with different concentrations of the growth factor to be tested. For other factors, the cells were plated in 6F for 24 h and then the growth factor under investigation was added at different concentrations in the presence of all other growth factors in 6F. Cell numbers were counted with a Coulter counter (Model Zf) after complete dispersion of the cells with trypsin-EDTA (Gibco-BRL).

Cell Differentiation Studies

Cells were plated in 6F at a 1:8 split in laminin-coated 24-well plates with F12/DMEM supplemented with 6F for 48 h and then washed with F12/DMEM and refed with fresh medium containing insulin, transferrin, and α-tocopherol. Growth factors such as bFGF (recombinant human bFGF, Gibco-BRL), aFGF (recombinant human aFGF, Gibco-BRL), forskolin (Calbiochem), NGF (7.5 S mouse NGF, Collaborative Research) were added individually or in combinations, as indicated. Medium was changed once after a 48 h incubation with the respective factors. Cultures were fixed for immunocytochemistry at the indicated time with ice-cold 1% glutaraldehyde (Ted Pella, Tustin, CA) in a 3% sucrose solution on ice for 30 min. The fixed cells were rinsed with ice-cold milli-Q water, treated with methanol containing 3% H_2O_2 for 30 min at room temperature and washed with PBS. The cells were then incubated with PBS containing 0.2% Triton X-100, 5% goat serum, and 0.1*M* NH_4HCO_3 for 1 h at 37°C. Primary antibodies (Table 3) were diluted in dilution buffer (PBS with 0.1% Triton X-100 and 1% BSA) and were added at 250 μL/well. Incubation with primary antibodies was carried out at 4°C overnight. Excess primary antibody was washed 5× with the dilution buffer and the cells were then incubated with appropriate enzyme-conjugated secondary antibodies (antimouse Ig F(ab)'-alkaliphosphatase, antimouse Ig F(ab)'-peroxidase and antirabbit IgG F(ab)'- peroxidase were purchased from Boehringer Mannheim) at 37°C for 60 min. The cells were washed 5× with dilution buffer and 2× with either 0.05*M* sodium acetate, pH 5.0, for peroxidase conjugates or with phosphatase substrate buffer. Specifically bound peroxidase activity was detected with DAB-H_2O_2 solution made using the Sigma peroxidase substrate kit. Alkaline phosphatase was detected with Boehringer alkaline phosphatase substrate (NTB/BCIP) diluted in substrate buffer. The color developing reaction was terminated by thoroughly rinsing with tap water and the specimens were preserved in glycerol/PBS (50:50). The stained samples were observed and micrographs were taken using a Nikon Diaphot bright field microscope. Dispersed neonatal rat brain cell cultures served as both positive and negtive controls and the specificity of the antibodies were comfirmed.

Immunofluorescence

For nestin immunofluorescence, cells grown on chamber slides were fixed *in situ* with 4% paraformaldehyde in phosphate buffer pH 7 for 30 min at room temperature. The fixed cells were washed 3× with PBS, blocked with 5% goat serum in PBS, 0.2% triton at 37°C for 60 min. Antinestin antiserum raised in rabbits against the last 1200 amino acids in the rat nestin sequence, which was expressed as a fusion protein with the N-terminus of TrpE, was kindly provided by R. McKay's laboratory. The antiserum was diluted at 1:1000 with dilution buffer and incubated with the cells at 4°C overnight. After 5× washes with dilution buffer (PBS with 0.1% Triton X-100 and 1% BSA), the samples were incubated with goat antirabbit IgG F(ab)'-FITC (Boehringer Mannheim) at 37°C for 1 h. The slides were washed 5× with dilution buffer and sealed in 50% glycerol in PBS. Specific immunofluorescence was observed and micrographs were taken under a Nikon epifluorescence microscope.

Western Blotting

Approximately 10^6 cells were directly lysed in 100 μL loading buffer and heated to 95°C for 5 min. The sample was chilled on ice and loaded onto a minigel (Novex precast SDS gel, 4–20%) The sample was fractionated by SDS gel electrophoresis at 75 mA until the front dye moved near the end. Electroblot transfer to nitrocellulose membrane was carried out in Tris-glycine-methanol buffer at 100 V for 1 h. The membrane was washed with water and was saturated by incubation with 1% BSA at 37°C with gentle shaking. Incubation of primary antibodies (anti-nestin antiserum 1:1000, antineuron specific enolase antiserum 1:1000) was carried out at 37°C for 2 h. Excess primary antibodies were washed out with three changes of incubation buffer, 10 min for each change. Then, the membrane was incubated with antirabbit IgG F(ab)'-peroxidase for 1 h. Finally, the membrane was stained with DAB-H_2O_2 as described above for immunocytochemistry.

Intracranial Injection of NEP Cells in Neonatal Rat Pups

NEP cells at passages between 35–40 were labeled with 5 μ*M* PKH-26 for 7 min at room temperature according to the protocol provided by the supplier (Sigma). Labeled cells were incubated with serum-free medium containing 100 ng/mL bFGF and 5 μ*M* forskolin at 37°C for 4 h. The cells were counted and a suspension of partially dissociated cells was prepared at a concentration of 5×10^7 cells/mL. An aliquot of 2 μL was injected into each side of the cerebellum or one side of the forebrain with a modified Hamilton syringe as described previously (Gao et al., 1991). Pups were returned to their mothers for 3–5 d. At the end of this time, injected animals were anesthetized with an overdose of ketamine, perfused with 4% paraformaldehyde, and

Table 3
Antibodies Used for Immunocytochemistry: Source and Titer

Antibodies	Source	Cat. no.	Titer
General neuron markers			
Neuron-specific enolase	Chemicon	MAB314	1 μg/mL
Tubulin-beta	Sigma	T4026	1:100
Microtubule ass. protein-2	Boehringer Mannheim	1284 959	2 μg/mL
Neurofilament 68 kDa	Sigma	N5139	1:40
Neurofilament 160 kDa	Sigma	N5264	1:40
Neurofilament 200 kDa	Sigma	N0142	1:400
Synaptophysin	Sigma	S5768	1:200
Tau	Chemicon	MAB361	1:100
Cortical neuron marker			
Protein kinase C-γ (1.12)	Boehringer Mannheim	1428 896	0.4 μg/mL
Neurotransmitters and synthesizing enzymes			
Glutamate	Chemicon	AB133	1:25,000
Aspartate	Chemicon	AB132	1:20,000
Serotonin	Chemicon	AB125	1:500
Choline acetyl-transferase	Boehringer Mannheim	1464 272	2 μg/mL
Tyrosine hydroxylase	Sigma	T1299	1:20,000
Oligodendricyte markers			
GalC	Boehringer Mannheim	1351 621	10 μg/mL
HNK-1	Sigma	C0678	1:2000
Astrocyte marker			
GFAP	Boeringer Mannheim	814 369	10 μg/mL
Peripheral nerve			
Peripherin	Chemicon	MAB1527	1:1000
General neural markers			
P75-LNGFR	Boehriniger Mannheim	1 198 645	5 μg/mL
Vimentin	Boehringer Mannheim	1112 457	10 μg/mL

brain tissues were dissected and postfixed with 4% paraformaldehyde at 4°C overnight. Brain sections of 100 μm thick were cut with a vibratome (model 1000) and observed under a Zeiss fluorescence microscope, a confocal microscope, and an EDGE 3-D fluorescent microscope. Photographs (Fig. 7) were taken with a Zeiss Axiophot fluorescence microscope and scanned into a Power Macintosh 8100/100 computer. The images were enhanced in contrast using the Adobe Photoshop program, aligned in composite and printed by Corporate Images.

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