Basic Fibroblast Growth Factor Increases Division and Delays Differentiation of Dopamine Precursors *in vitro*

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Growth factors have been shown to be involved in the regulation of division and differentiation of neuroepithelial cells. In the present study we examined the ability of various factors to influence the development of dopamine precursors. Primary neuronal cultures were prepared from the embryonic day 12 (E12) rat ventral mesencephalon, a time and place which coincides with the beginning of the birth of the dopamine neurons of the substantia nigra pars compacta. At low plating density in serum-free medium, the dopamine precursors divide for approximately 1 d *in vitro*. We report here that basic fibroblast growth factor (bFGF) can expand the period of dopamine precursor division at least until day 8 in culture, which is well beyond the normal division of these cells. This increase in cell division was accompanied by a delay in differentiation as compared to untreated control cultures. Upon differentiation, the high-affinity dopamine uptake values in bFGF-treated cultures were 20 times maximal control values. Mature dopamine neurons appeared at the same time as astrocytes, which may be playing a role in inducing dopamine neuron differentiation. IGF-I, GDNF, and EGF were unable to mimic the effect of bFGF on division and differentiation of dopamine precursors. Expanding *in vitro* the number of dopamine precursors provides tissue that may be suitable for transplantation in patients with Parkinson's disease.

[Key words: basic fibroblast growth factor, dopamine, precursor, transplantation, mitosis, mesencephalon, astrocyte, cell culture, differentiation]

During embryonic development, cells that give rise to the CNS proliferate in the ventricular zone of the neural tube. In the rat embryo, cells destined to become the substantia nigra are produced in the rostral portion of the ventral mesencephalon from embryonic day 11 (E11) to E15 (Hanaway et al., 1972; Lauder and Bloom, 1974). Mesencephalic dopamine neurons begin to differentiate at E12.5 (Specht et al., 1981) as demonstrated by the appearance of tyrosine hydroxylase (TH), the rate limiting enzyme in the synthetic pathway of dopamine. Although the division and differentiation of neuroepithelial cells follows a fixed schedule *in vivo*, it has been shown that *in vitro* the period

of division can be expanded (Temple, 1989) and the process of differentiation can be altered (Banker and Cowan, 1977).

Several growth factors have been shown to affect the survival and division of neuroepithelial cells *in vitro*. For example, epidermal growth factor (EGF) allows the survival and proliferation of precursor cells from E16 rat mesencephalon (Mytilineou et al., 1992; Casper et al., 1991) and adult mouse striatum (Reynolds and Weiss, 1992). EGF, as well as basic fibroblast growth factor (bFGF), stimulates cell proliferation and increases dopamine uptake in cultures of E15–17 rat mesencephalon (Knusel et al., 1990; Park and Mytilineou, 1992). bFGF has been shown to improve the growth and survival of dopamine neurons *in vitro* (Ferrari et al., 1989; Engele and Bohn, 1991). A number of studies indicate that bFGF is mitogenic to neuronal precursors (Gensburger et al., 1987; Murphy et al., 1990; Ray et al., 1993) and the mRNA for the bFGF receptor is found in the epithelium lining the ventricles of the rat embryonic CNS (Wanaka et al., 1991), where mitotic precursor cells are found. In addition, other growth factors in conjunction with bFGF have been shown to stimulate proliferation of neuronal precursors *in vitro* in a number of systems. For example, neuronal precursors from E14 rat striatum proliferate in response to bFGF and NGF (Cattaneo and McKay, 1990). bFGF and insulin-like growth factors (IGFs) induce proliferation of precursors in E10 mouse neuroepithelium (Drago et al., 1991), and E10 mouse mesencephalon can be induced to proliferate with bFGF and serum (Kilpatrick and Bartlett, 1993).

In the present study we investigated the regulation of division and differentiation of dopamine precursors in the ventral mesencephalon. In order to start with the maximum number of dopamine precursors, we used ventral mesencephalon from E12 rat, the developmental stage that corresponds to the beginning of the birth of the dopamine neurons of the substantia nigra. Cells were cultured in serum-free media in the presence of various growth factors in an attempt to stimulate the division of neuroepithelial cells. We report that bFGF extends the time over which dopamine precursors divide *in vitro*. The delay in differentiation and continuance of cell division results in a larger number of dopamine neurons, with bFGF-treated cultures achieving high-affinity dopamine uptake values approximately 20 times higher than values from untreated control cultures.

Materials and Methods

Preparation of cell cultures. Pregnant Sprague-Dawley rats at 12 days gestation (embryonic day 12 = E12; Taconic Farms, Taconic, NY) were sacrificed, and the embryos (crown-rump length = 8 mm) removed. The ventral mesencephalon was dissected out (see Fig. 1) without the membrane coverings and pooled on ice in Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS). Tissue was suspended in a chemically defined medium, which was composed of a basal medium (Eagle's MEM

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Figure 1. Photomicrograph of an E12 rat embryo. Black lines indicate the location of the rostral and caudal incisions, and the dotted line the incision made to isolate the ventral mesencephalon. Scale bar, 1 mm.

and HAM F-12 nutrient mixture 1:1, GIBCO, Grand Island, NY) containing glucose (33 mM), glutamine (2 mM), and HEPES buffer (15 mM), as well as the supplements of N2 described by Bottenstein and Sato (1979): transferrin, 100 μ g/ml; insulin, 25 μ g/ml; progesterone, 20 nM; putrescine, 60 μ M; sodium selenite, 30 nM. The tissue was gently triturated through a fire-polished pipette, and the resulting cell suspension was plated at 18,000 cells per cm^2 in 35 mm plastic dishes (Falcon, Lincoln Park, NJ) pre-coated with polyornithine (100 μ g/ml, Sigma, St. Louis, MO). The final volume in the dishes was 1.5 ml, and 1 ml was replaced with fresh media three times a week. The cultures were maintained in an incubator at 37°C, in an atmosphere of 95% air and 5% CO_2 , 100% relative humidity. Growth factors were added at the time of plating and at each feeding thereafter. bFGF (human recombinant; Synergen, Boulder, CO), EGF (mouse submaxillary gland; Collaborative Research, Lexington, MA), and IGF-I (human recombinant; GIBCO, Grand Island, NY) were used at a concentration of 20 ng/ml. Glial cell line-derived neurotrophic factor (human recombinant GDNF; Synergen, Boulder, CO) was used at a concentration of 1 ng/ml.

³H-Thymidine incorporation. ³H-Thymidine incorporation was measured by a modification of the method described by Simpson et al. (1982). Cultures were exposed to 2 μ Ci/ml ³H-thymidine (New England Nuclear, Boston, MA) for 24 hr at 37°C, in the presence of growth factors as appropriate. Cultures designated as blanks received 1000-fold cold thymidine in addition to the radioactively labeled thymidine. After washing three times with basal media, the cultures were treated with 10% trichloroacetic acid (TCA) for 30 min at 4°C, to remove free ³H-thymidine. The cultures were then rinsed three times with 10% TCA, and once with 95% ethanol. The incorporated ³H-thymidine was solubilized with 0.5 M NaOH for 30 min at 37°C, which was then neutralized with 1 M HCl; 1 ml of this solution was added to 10 ml of scintillation cocktail (Ecoscint; National Diagnostics, Mannville, NJ) and counted in a scintillation spectrometer.

Immunocytochemistry. Cultures were fixed in 10% formalin, 0.03% glutaraldehyde in 0.1 M sodium acetate buffer, pH 6.5, for 10 min followed by 10% formalin, 0.03% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 8.5, for 1 hr at room temperature (O'Malley et al., 1991). Neurons were labeled with tau (polyclonal from Sigma, St. Louis, MO) which *in vitro* labels the soma and processes (Kosik and Finch, 1987). Astrocytes were labeled with antibodies to glial fibrillary acidic protein (GFAP; polyclonal from Accurate, Westbury, NY), and dopamine neurons were labeled with antibodies to tyrosine hydroxylase (TH; monoclonal from INCSTAR, Stillwater, MN; polyclonal from Eugene Tech, Richfield Park, NJ). Cells were permeabilized with 0.2% Triton X-100 for 30 min, and reacted with antibodies at the recommended dilutions overnight at 4°C. They were visualized with the peroxidase- or alkaline phosphatase-coupled avidin-biotin staining kit (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) with diaminobenzidine (DAB) or Vector red as a chromagen.

To label dividing dopamine precursors, cultures were pulsed for 24 hours with 1 μ M BrdU at DIV 1–2, 4–5, or 8–9, then fixed on DIV 12. Cultures that were pulsed on DIV 12–13 were fixed on DIV 16. The cells were fixed with 4% paraformaldehyde for 30 min, treated with 2N HCl, neutralized with borate buffer (pH 8.5), then reacted with BrdU

Figure 2. Thymidine incorporation in control and bFGF (20 ng/ml) treated cultures exposed to ³H-thymidine (2 μ Ci/ml) for 24 hr at various times *in vitro*. The bars represent the means $\pm$ SEM of 10–20 samples per group from five separate experiments. bFGF-treated cultures incorporated significantly higher amounts of ³H-thymidine at all time points ($p < 0.001$; unpaired *t* test).

antibodies overnight (mouse monoclonal from Becton-Dickinson, San Jose, CA). Staining proceeded using the peroxidase-coupled avidin-biotin staining kit (Vector Labs) with DAB as a chromagen and cobalt chloride and nickel ammonium sulfate as intensifiers. Cells were double labeled with TH (monoclonal from INCSTAR, Stillwater, MN) and visualized using the peroxidase-coupled avidin-biotin staining kit with 3-amino-9-ethylcarbazole (AEC) substrate kit (Vector Labs) as a chromagen.

To label other dividing cell types, cultures were pulsed with 5-bromo-2'-deoxyuridine (BrdU; Amersham, Arlington Heights, IL) for 24 hr at 10 μ M. Cells were fixed in 90% ethanol, 5% glacial acetic acid, and 5% water, reacted with antibodies to BrdU (monoclonal, Amersham), and visualized with the peroxidase-coupled staining kit (Cell Proliferation Kit, Amersham) with DAB as a chromagen. Double labeling was achieved using the antibodies described above, visualized with the alkaline phosphatase-coupled avidin-biotin staining kit (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) with Vector red as a chromagen.

³H-Dopamine uptake. Cultures were incubated for 30 min at 37°C with ³H-dopamine (0.5 μ Ci/ml, 40 Ci/mmol, New England Nuclear, Boston, MA) in a Krebs Ringer phosphate buffer (0.1 M) with added EDTA (1.3 mM), glucose (5.6 mM) and ascorbic acid (0.2 mg ml^{-1}). Cells were then rinsed twice with buffer, incubated for 30 min at 37°C with 95% ethanol, which was then added to 10 ml of scintillation cocktail (Ecoscint; National Diagnostics, Mannville, NJ) and counted. Counts were corrected for nonspecific uptake using cultures that were treated with the dopamine uptake blocker mazindol (10 μ M; Sandoz Pharmaceuticals, Hanover, NJ).

Results

Effect of bFGF on the division and differentiation of embryonic mesencephalic cells

The effect of bFGF on the proliferation of cells derived from E12 rat ventral mesencephalon was examined. ³H-Thymidine *in*

Figure 3. Photomicrographs from bFGF-treated cultures immunostained with antibodies to GFAP (*A* and *B*) or tau (*C* and *D*). Only very few faint GFAP-positive processes are present on DIV 5 (*A*, arrowheads), but many astrocytes are labeled with GFAP antibodies on DIV 9 (*B*). Tau-positive process-bearing neurons aggregated in a cluster on DIV 5 (*C*). The clusters increase in size by DIV 9. Scale bar, 25 μ m.

corporation during a 24 hr period was used as an index of total cell division. Preliminary experiments using 0.1, 1.0, 10, and 100 ng/ml bFGF showed that the effect peaked at approximately 10–20 ng/ml. All subsequent experiments were done using 20 ng/ml bFGF added three times a week. Figure 2 shows that incorporation in control cultures was highest after 1–2 d *in vitro* (DIV), and then declined until DIV 8–9, indicating a limited mitotic activity of mesencephalic cells during the first few days *in vitro*. The presence of bFGF in the cultures resulted in a significant increase in 3 H-thymidine incorporation over control values at all time points examined. Incorporation in the bFGF-treated cultures continued to increase throughout 13 d *in vitro*, with the highest rate of increase occurring between DIV 1–2 and DIV 4–5 (2.3-fold increase).

Immunocytochemistry was performed on DIV 5, 9 and 13, to determine the phenotype of the cells that were generated by bFGF-induced cell proliferation. Antibodies against the intermediate filament glial fibrillary acidic protein (GFAP) were used as markers for astrocytes. Neurons were labeled with antibodies against the microtubule-associated protein tau, which *in vitro* is present in the soma and processes (Kosik and Finch, 1987). After 5 d *in vitro* very few GFAP-positive astrocytes could be observed in cultures treated with bFGF (Fig. 3*A*). This finding indicates that the 2.3-fold increase in 3 H-thymidine incorporation between DIV 1–2 and 4–5 (Fig. 2) was not due to proliferation of differentiated astrocytes. The presence of differentiated glia would not be expected in the first four d in cultures prepared

from E12 rat embryos, since glial differentiation *in vivo* begins at E16 and dissociated cultures taken after E10 show a parallel development *in vitro* (Abney et al., 1981). Nonetheless, the number of GFAP-positive astrocytes increased greatly in bFGF-treated cultures by DIV 9 (Fig. 3*B*), and even further by DIV 13 (not shown). By contrast in control cultures virtually no GFAP-positive astrocytes could be found at DIV 5, 9, or 13, as expected given that a low plating density and serum-free media do not support the growth of astrocytes (Walicke and Baird, 1988). Tau-positive neurons mainly in the form of clusters could be seen on DIV 5 in cultures treated with bFGF (Fig. 3*C*) and the size of these tau-positive clusters increased with time, indicating an increase in the overall number of differentiated neurons (Fig. 3*D*). In control cultures, tau-positive neurons were also found primarily in clusters, but their size was smaller than those treated with bFGF and the size did not appear to increase over time. In addition, beginning on DIV 9 in control cultures some clusters containing tau-positive neurons appeared to be in the process of degeneration, as indicated by the presence of broken-up neurites. Degenerating clusters were not detected in the bFGF-treated cultures, indicating a survival effect of bFGF.

The presence of dopamine neurons was visualized by staining with antibodies against TH. In control cultures at DIV 5, there were a number of strongly stained cells (Fig. 4*A*). In contrast, bFGF-treated cultures at DIV 5 had only a few very immature TH-positive cells (Fig. 4*C*) suggesting that bFGF delayed the differentiation of dopamine neurons. By DIV 9 there was growth

Figure 4. Dopamine neurons from control (*A, B*) and bFGF-treated cultures (*C–E*) labeled with antibodies to TH. In control cultures, TH labeled neurons are present on DIV 5 and their processes increase in length and complexity by DIV 9. Indication of degenerating processes is also seen at this time (*arrowhead*). Very few, immature TH-positive neurons are found in bFGF-treated cultures on DIV 5 (*arrowheads*). By DIV 9, the number of TH-positive neurons and their processes have increased greatly (*D*). A micrograph taken at lower magnification (*E*) shows the size of the cluster and the extent of process outgrowth of TH-positive neurons. Magnification for *A–D*, bar = 25 μm ; for *E*, bar = 100 μm .

of TH-positive processes in control cultures but many of the TH-positive cells began to show signs of degeneration (Fig. 4*B*), indicating limited survival of this cell type. On the other hand, much larger clusters with many well differentiated TH-positive cells could be observed on DIV 9 in cultures treated with bFGF (Fig. 4*D,E*).

Because of the clustering and overlapping of the cells, counting of the number of TH-positive neurons in the cultures was not feasible. However, the presence of dopamine neurons was

quantitated by measuring ^3H -dopamine uptake, which reflects the number of dopamine neurons and the extent of neuritic growth. Uptake in control cultures increased between DIV 2 and 9 (Fig. 5), but it was reduced on DIV 13, probably as a result of the deterioration of the overall cell survival in these cultures. The bFGF-treated cultures had lower uptake levels than the controls at DIV 2 and 5 (Fig. 5), which agreed with immunocytochemical results showing delayed differentiation of the TH-positive neurons on DIV 5 (Fig. 4). Between DIV 5 and 9 there

Figure 5. ^3H -Dopamine uptake in control and bFGF-treated cultures during development *in vitro*. The bars represent the means $\pm$ SEM of four samples per group (representative of three experiments). ^3H -Dopamine uptake in bFGF-treated cultures at DIV 5 was significantly lower than in the controls ($p < 0.001$), while on DIV 9 and 13 it was significantly higher ($p < 0.001$; unpaired t test).

was a 150-fold increase in ^3H -dopamine uptake in the bFGF-treated cultures, which again confirmed the results obtained by TH-immunocytochemistry showing increased number and maturation of dopamine neurons. A further but slower increase in dopamine uptake occurred on DIV 13, reaching a value approximately 20 times the maximal control value.

The increase in the number of TH-positive neurons observed between DIV 5 and DIV 9 in the bFGF-treated cultures could be the result of proliferating dopamine precursors. To determine whether precursors of dopamine neurons were dividing in cultures treated with bFGF, we labeled dividing cells *in vitro* by pulsing the cultures with the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) for 24 hr on DIV 1–2, 4–5, 8–9, and 12–13. The cultures were grown further to allow differentiation of the pulsed cells and then processed for double labeling with antibodies against BrdU in conjunction with TH. Cells double labeled for BrdU and TH were observed in cultures pulsed on DIV 1–2, DIV 4–5, and DIV 8–9 and allowed to grow until DIV 12 (Fig. 6). Of the cells that were TH+ at DIV 12, the percent that were double labeled at each time point was quantitated (Table 1). 31.5% of the cells were dividing at DIV 1–2. The largest number of double labeled cells were seen in cultures pulsed on DIV 4–5, where 74.5% of the TH-positive cells had some degree of BrdU labeling in the nucleus (Fig. 6B–E). Double labeled cells could also be seen in cultures pulsed on DIV 8–9 (Fig. 6F), but their number had decreased to 8.7%. No double labeled cells could be observed when cells were pulsed on DIV 12–13 and fixed on DIV 16. This staining indicates that dopamine pre-

cursors in the bFGF-treated cultures were dividing at least up until DIV 8.

Double labeling with antibodies to BrdU and tau or GFAP was also performed in cultures pulsed on DIV 1–2, 4–5, 8–9, and 12–13, to determine whether neuronal and astroglial precursors were dividing across all time points. Cultures pulsed with BrdU on DIV 1–2 and fixed on DIV 12 had many tau-positive neurons double labeled with BrdU, but no double labeled astrocytes. When the BrdU pulse was on DIV 4–5, the number of double labeled tau-positive neurons was greater than those pulsed on DIV 1–2. Some GFAP-positive astrocytes were also double labeled at this time. Double labeled neurons were less frequently observed when BrdU was added on DIV 8–9 or 12–13. In contrast, about 50% of the GFAP-positive astrocytes were double labeled when the cultures were pulsed with BrdU on DIV 8–9 or 12–13, suggesting active glial proliferation at these time points.

Influence of other growth factors on the division and differentiation of embryonic mesencephalic cells

The effect of the continuous presence of other growth factors on the development of mesencephalic cells was examined. In addition to bFGF, both EGF and IGF-I have been shown to be mitogenic to mesencephalic precursors (Lenoir and Honegger, 1983; Mytilineou et al., 1992), and GDNF has been shown to promote the survival and differentiation of dopamine neurons in rat embryonic midbrain cultures (Lin et al., 1993). The effect on total cell division of these factors, either alone or in conjunction with bFGF, was examined using ^3H -thymidine incorporation at DIV 4–5 (Fig. 7). Only bFGF increased the rate of cell division in E12 mesencephalic cultures. Treatment with the other growth factors yielded values that resembled those from controls, and did not modify the bFGF effect in cotreated cultures. The effect of the continuous presence of GDNF, EGF, and IGF-I on the ^3H -dopamine uptake in these cultures at DIV 5 and 9 was also examined (Fig. 8). None of the growth factors alone were able to mimic the ability of bFGF to either delay differentiation at DIV 5 or yield high uptake values at DIV 9. Although there was a small increase at DIV 9 in the EGF, GDNF, and IGF-I conditions, this increase did not differ significantly from control values (two-tailed t test). The growth factors were also unable to modify the bFGF effect in cotreated cultures.

The ability of various factors to affect the differentiation of dopamine precursors generated by treatment with bFGF was also examined (Table 2). In addition to GDNF, retinoic acid and dibutyryl cyclic adenosine monophosphate (dbcAMP) were used because they have been shown to stimulate neuronal differentiation in rodent embryonic cultures (Skolnik-Tavron and Segal, 1993; Ved and Pieringer, 1993). Cultures were grown for 5 d in the presence of bFGF (at which time ^3H -dopamine uptake was measured to obtain a baseline value of dopamine neuron development). bFGF was then washed off the cultures, and either retinoic acid (0.1 μM), dbcAMP (1 mM), GDNF (1 ng/ml), or no differentiation factor was added in the presence or absence of bFGF. ^3H -Dopamine uptake was performed on all cultures at DIV 9. The data show that none of the factors was able to significantly increase the dopamine uptake in these cultures, nor were they able to modify the bFGF effect in cotreated cultures. Interestingly, there was also no significant difference in the development of dopamine neurons on DIV 9 between cultures treated continuously with bFGF and cultures treated with bFGF only until DIV 5. This finding indicates that the effect of bFGF

Figure 6. Photomicrographs from cultures labeled with antibodies to BrdU (black) and TH (red). BrdU labeled nuclei indicate that the cells were in the S phase of the mitotic cycle during the time of BrdU exposure (A, DIV 1–2; B–E, DIV 4–5; and F, DIV 8–9). All cultures were fixed on DIV 12. Double labeling with BrdU and TH indicates that these dopamine neurons differentiated from precursors that were still dividing during BrdU exposure. Scale bar, 20 μ m.

on dopamine neurons occurs in large part during the first 5 d in culture.

Discussion

The dopamine neurons of the rat substantia nigra are generated from dividing neuroepithelial cells in the ventral mesencephalon during a span of about 5 d, between E11 and E15 (Hanaway et al., 1972; Lauder and Bloom, 1974; Altman and Bayer 1981). Expression of phenotypic markers begins after the final mitosis (Rothman et al., 1980; Specht et al., 1981) with TH being detectable first at E12.5 (Specht et al., 1981). Differentiation of dopamine neurons proceeds quickly and axonal processes have already assembled in the medial forebrain bundle and reached

the ventral-lateral portion of the neostriatum by E14.5 (Specht et al., 1981).

It has previously been shown that bFGF and serum can increase mitosis during the normal period of cell division of dopamine precursors (about 1 d *in vitro*) from E12 rat embryos (Mayer et al., 1993). We now report that the period of proliferation of dopamine precursors can be extended to at least 8 d *in vitro* by the continuous exposure of E12 ventral mesencephalic cells to bFGF in serum-free medium. Incorporation of the thymidine analogue BrdU followed by double labeling with antibodies to BrdU and TH demonstrated that precursors of dopamine neurons divide for at least 8 d *in vitro*. In the embryo this would correspond to E20, well beyond the end of the birth

Table 1. Quantitation of *in vitro* division of dopamine precursors

BrdU pulse (DIV)	% Double labeled TH+ cells	Total number of TH+ cells examined
1–2	31.5 ± 1.8	574
4–5	74.5 ± 2.7	501
8–9	8.7 ± 1.7	510
12–13	0	330

Cultures were pulsed for 24 hr with 1 μ M BrdU at DIV 1–2, 4–5, 8–9, or 12–13. Cultures were further grown to allow differentiation, then fixed and processed for TH/BrdU double label immunocytochemistry. The number of double labeled cells was expressed as a percentage of the total number of TH+ cells counted in each dish ($N = 5$ dishes for DIV 1–2, 4–5, and 8–9 and $N = 3$ dishes for DIV 12–13). Numbers represent the mean $\pm$ SEM. The largest number of dopamine precursors was dividing on DIV 4–5.

period of dopamine neurons of the substantia nigra. Quantitation of these double labeled cells indicated that, of the cells that were TH+ at DIV 12, approximately three-quarters of them had been dividing at DIV 4–5. The stimulation of cell division by bFGF is accompanied by an initial delay in the differentiation of dopamine neurons. When no growth factors were added, maturation of dopamine neurons by DIV 2 and continuing through DIV 9 was indicated by increased 3 H-dopamine uptake, and by increased process outgrowth in TH immunolabeled cells. By comparison, in the presence of bFGF the numbers of TH-positive neurons, their process outgrowth, and their ability to accumulate 3 H-dopamine were minimally developed on DIV 2 and DIV 5. Between DIV 5 and DIV 9, however, differentiation began to occur at a very rapid rate in bFGF-treated cultures, causing the appearance of many TH-positive neurons by DIV 9 with well developed processes and a 3 H-dopamine uptake level which reached 150 times that of DIV 5. These results indicate that bFGF can delay the terminal differentiation of dopamine precursors and prolong their period of mitosis *in vitro*, with the consequence of an increased number of differentiated dopamine neurons at later time points.

The stimulation of proliferation by bFGF did not appear to be restricted to dopamine precursors. Neurons labeled with antibodies to tau increased in cultures treated with bFGF and a large proportion of the cells that were tau-positive at DIV 12 had incorporated BrdU during the first 5 d in culture. It is known that the cells destined to become the GABA neurons of the substantia nigra pars reticulata have the same birthdates and originate from the same area of the neuroepithelium as the dopamine neurons of the pars compacta (Hanaway et al., 1972; Altman and Bayer, 1981). Therefore, it is likely that both types of precursors are present in our cultures and are dividing in response to bFGF, since there is evidence that bFGF is mitogenic to GABA precursors (Deloulme et al., 1991) and that bFGF increases the survival and neurite outgrowth of GABA neurons in cultures from E14 rat mesencephalon (Ferrari et al., 1989, 1991).

Stimulation of cell proliferation by bFGF during the first days in culture involved mainly neuronal precursors. Although bFGF is a potent mitogen of astrocytes (Kniss and Burry, 1988; Park and Mytilineou, 1992), staining with the astrocytic marker GFAP showed no differentiated astrocytes in the cultures treated with bFGF during the first 5 d *in vitro*. Furthermore, in cultures pulsed with BrdU on DIV 1–2 and fixed on DIV 12, no BrdU labeled GFAP-positive astrocytes could be found, suggesting that the cells dividing on DIV 1–2 did not include precursors of

Figure 7. Effect of various growth factors on 3 H-thymidine incorporation in E12 mesencephalic cultures during DIV 4–5. Bars represent the means $\pm$ SEM of 12–13 samples from three separate experiments. Only bFGF was effective in stimulating cell proliferation in the cultures ($p < 0.001$; analysis of variance followed by Tukey test). Cotreatment with bFGF and the other growth factors did not modify the effect of bFGF.

astrocytes. Some double labeled astrocytes could be observed when BrdU was added on DIV 4–5, but numerous proliferating astrocytes incorporated BrdU on DIV 8–9 and 12–13. Whether bFGF stimulated the proliferation of glial precursors or differentiated astrocytes at these later time points cannot be determined from these data. However, a large number of differentiated astrocytes is already present on DIV 9, and these cells are probably responding to the mitogenic effects of bFGF.

The stimulation of precursor cell division followed by increased differentiation of dopamine neurons was specific to bFGF. Although both EGF and IGF-I can stimulate the proliferation of neuronal precursors (DiCicco-Bloom et al., 1990; Mytilineou et al., 1992; Reynolds and Weiss, 1992), they showed no mitogenic effect on E12 mesencephalic cells, and were not able to modify the mitogenic effects of bFGF. It is possible that receptors for these growth factors are not present or are not biologically active on these cells during this stage of development. GDNF, a recently characterized growth factor (Lin et al., 1993) with potent neurotrophic activity for dopamine neurons, was also unable to stimulate cell proliferation. Furthermore, EGF, IGF-I and GDNF failed to mimic the bFGF effect on differentiation of dopamine precursors and were unable to modify the bFGF effect in cotreated cultures.

It is of interest that the rapid rate of differentiation and growth of dopamine neurons, which occurs between DIV 5 and DIV 9, coincides in time with the appearance of astrocytes in the cultures. As stated, bFGF is mitogenic to astrocytes (Kniss and Burry, 1988). In turn it is well established that astrocytes possess neurotrophic activity (Banker, 1980; Müller and Siefert, 1982; Kadle et al., 1988) and promote the survival and development of dopamine neurons (Beyer et al., 1991; O'Malley et al., 1994). It has also been shown that treatment of cortical astrocytes with bFGF induces the secretion of molecules which stimulate the differentiation of mesencephalic dopamine neurons (Gaul and Lubbert, 1992). It seems quite possible that the bFGF treatment is causing division of astrocytes, and that the resulting large number of astrocytes is inducing the differentiation of dopamine neurons.

Figure 8. Effect of growth factors on the development of ^3H -dopamine uptake in E12 mesencephalic cultures. The bars represent the means $\pm$ SEM of 10–14 samples from three separate experiments. Treatment with bFGF resulted in significantly lower uptake values on DIV 5 compared to the controls ($p < 0.001$) and higher on DIV 9 ($p < 0.001$; Analysis of variance followed by Tukey test). This effect was not modified by cotreatment with other growth factors. GDNF, EGF, and IGF-I were ineffective in modifying ^3H -dopamine uptake on DIV 5. Although the values at DIV 9 were higher than control in GDNF, EGF, and IGF-I treated cultures, they did not differ significantly (two-tailed t test).

Recently it has been shown that the induction of dopamine neurons in the ventral mesencephalon occurs through contact with the floor plate (Hynes et al., 1995), and the authors suggest that the induction of neurons in this area may be the result of both diffusible and contact-dependent signals. Within the context of our results, it is possible that in addition to stimulating division of dopamine precursors, bFGF may be stimulating the production of a dopamine neuron inducer, which would also contribute to the increase in dopamine neurons seen at later time points.

Neuronal precursors generated during the first 5 d of treatment with bFGF differentiated into dopamine neurons even if bFGF was removed on DIV 5. This suggests that the actions of bFGF, which resulted in an increased number of differentiated dopamine neurons on DIV 9, occurred in large part during the first five d. One explanation for this finding is that an initial exposure to bFGF is sufficient to set in motion the extended proliferation of these cells. Alternatively, perhaps the appearance of mature astrocytes between DIV 5 and 9 is stimulating the differentiation of dopamine precursors, gradually bringing the period of division to an end.

Whether all dopamine neurons present in the bFGF-treated cultures on DIV 9 were generated from precursor cells dividing *in vitro* cannot be determined from our data. Some precursor cells which had undergone their last division *in vivo* could have been maintained in an undifferentiated state by bFGF during the first 5 d *in vitro*, and bFGF could be supporting their survival. However, as shown by the incorporation of BrdU, a large number of the dopamine neurons underwent their last division *in*

vitro. Thus either precursors were already committed to the dopamine phenotype or culture conditions were favorable for the expression of dopaminergic traits, since commitment to phenotype can be determined by both intrinsic programs and epigenetic signals (Temple, 1989; Luskin et al., 1993; Hynes et al., 1995).

Parkinson's disease is a neurodegenerative disorder characterized by the loss of dopamine neurons of the substantia nigra pars compacta. A form of therapy for this disease is the transplantation of embryonic mesencephalic cells into the denervated striatum, although the limited efficacy of this treatment is indicative of the need for further research in this field (for a review, see Björklund, 1991). The requirement to have available enough fetal tissue of the appropriate developmental stage at the time of transplantation and the poor survival of the grafted cells within the host brain are major difficulties with this treatment. The *in vitro* manipulation of dopamine precursors could result in a cell population appropriate for transplantation. This would create a window of time between collection of fetal tissue and grafting. Furthermore, a cell population rich in dopamine precursors may survive and integrate better than differentiated neurons.

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Table 2. Effect of neuronal differentiation factors on the development of dopamine neurons in E12 mesencephalic cultures treated with bFGF for 5 DIV

Differentiation factors added on DIV 5	^3H -Dopamine uptake on DIV 9 (pmol/culture/30 min)	
	(+) bFGF	(-) bFGF
None	1.73 $\pm$ 0.11	1.76 $\pm$ 0.12
Retinoic acid (0.1 μM)	2.30 $\pm$ 0.22	1.82 $\pm$ 0.08
GDNF (1 ng ml $^{-1}$)	2.03 $\pm$ 0.20	1.96 $\pm$ 0.20
dbcAMP (1 mM)	1.83 $\pm$ 0.21	2.01 $\pm$ 0.13

Values are means $\pm$ SEM from four cultures per group. There was no significant difference between the groups. Cultures were treated with 20 ng/ml bFGF until DIV 5 and then the differentiation factors were added in the presence or absence of bFGF. ^3H -Dopamine uptake in cultures treated with bFGF for 5 DIV was 0.69 ± 0.16 pmol/culture/30 min.

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