The Neurotrophic Effects of Fibroblast Growth Factors on Dopaminergic Neurons *in vitro* Are Mediated by Mesencephalic Glia

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Neurotrophic support is generally believed to result from a direct action of growth factors on developing neurons. However, there is increasing evidence that growth factors can indirectly affect neuronal development by glial-mediated processes. To investigate a possible role of glia in mediating neurotrophic effects on dopaminergic neurons, four purified growth factors were screened for dual effects on the survival and differentiation of dopaminergic neurons and on the proliferation of mesencephalic glial cells *in vitro*. Dissociated embryonic day 14.5 rat mesencephalon was grown at low cell density without serum, conditions under which both glial growth and neuronal survival are not optimal. Treatment of these cultures with acidic fibroblast growth factor (aFGF) or basic fibroblast growth factor (bFGF) increased the number of surviving tyrosine hydroxylase–immunoreactive (TH-IR) neurons by 190–210% at 8 d *in vitro* in a dose-dependent manner. The effects of these factors were not additive. High-affinity dopamine uptake was increased by bFGF, but not by aFGF. Length of TH-IR neurites was not affected by either aFGF or bFGF.

Both growth factors induced proliferation of mesencephalic astrocytes as demonstrated by autoradiographic labeling with ^{3}H-thymidine combined with immunocytochemistry for glial fibrillary acidic protein (GFAP). In contrast, platelet-derived growth factor (PDGF) and interleukin-1 had no effect on the survival or differentiation of dopaminergic neurons or the proliferation of mesencephalic astrocytes. Inhibition of glial proliferation abolished the neurotrophic effects exerted by aFGF or bFGF on dopaminergic neurons. Moreover, conditioned medium derived from mesencephalic glial cultures replated in the virtual absence of neurons also contained neurotrophic activity.

These observations provide direct evidence that mesencephalic astrocyte precursors are targets for aFGF and bFGF and that these glia provide neurotrophic factors for central dopaminergic neurons.

The majority of neurotrophic factors are small polypeptide molecules that act on specific classes of neurons to support survival and differentiation during development, and survival and re-

generation in the adult (for review, see Thoenen et al., 1987). In the CNS, the only neurotrophic actions sufficiently characterized are those of β-NGF on cholinergic neurons of the basal forebrain (for reviews, see Whittemore and Seiger, 1987; Hefti et al., 1989). However, there is increasing evidence that other known growth factors, such as fibroblast growth factor (FGF), affect survival and differentiation of a variety of central neurons (Morrison et al., 1986; Walicke, 1988; Torres-Aleman et al., 1990). *In vitro* studies have shown that glial cells provide neurotrophic support for central neurons by releasing yet-unidentified substances (Lindsay, 1979; Banker, 1980; Manthorpe et al., 1981; Barbin et al., 1984; Hatanaka and Tsukui, 1986). Moreover, proliferation and differentiation of glia have been found to be affected by a variety of growth factors, among them basic FGF (bFGF) and acidic FGF (aFGF; Pruss et al., 1982; Hatten et al., 1988; Morrison et al., 1988), platelet-derived growth factor (PDGF; Richardson et al., 1988), and interleukin-1 (Giulian et al., 1988).

Previous studies have demonstrated that mesencephalic glia specifically support the survival of dopaminergic neurons (O'Malley et al., 1989) and induce dendritic outgrowth (Chamak et al., 1987). Knusel et al. (1990) recently reported that a stimulation of dopaminergic cell differentiation by epidermal growth factor (EGF) and bFGF can be abolished by blocking cell proliferation. Similarly, our studies have shown that the neurotrophic effects on dopaminergic neurons exerted by conditioned media produced by several glial tumor cell lines are paralleled by an increase in resident mesencephalic astrocytes (Engele et al., 1991).

In the present study, we further examined the possibility that growth factors affect dopaminergic cell development through effects on mesencephalic glia. aFGF, bFGF, PDGF, and interleukin-1 were screened for effects on development of dopaminergic neurons and proliferation of glia in serum-free, low-density cultures of the embryonic mesencephalon. The role of glial cells in putative neurotrophic effects was characterized by testing (1) growth factors for their survival-promoting effects in mesencephalic cultures with inhibited glial growth and (2) conditioned medium derived from mesencephalic glia for neurotrophic effects on dopaminergic neurons. Effects of the various treatments on the numbers of dopaminergic neurons and astrocytes were assessed by counting tyrosine hydroxylase–immunoreactive (TH-IR) and glial fibrillary acidic protein–immunoreactive (GFAP-IR) cells, respectively. Effects on the differentiation of dopaminergic neurons were assessed by determining neurite length and high-affinity dopamine uptake (Engele et al., 1989). Effects of growth factors on cell proliferation were studied by labeling cultures with ^{3}H-thymidine.

Materials and Methods

Mesencephalic assay cultures. For establishing mesencephalic assay cultures, two-thirds of the rostral mesencephalon was excised from embryonic day 14.5 (E14.5) Sprague–Dawley rats (Charles River, Kingston; 56–62 somites), trypsinized (0.1%) for 20 min, and dissociated by trituration. Cells were pelleted at 400 × g for 10 min, resuspended in 90% minimum essential medium (MEM; Gibco) and 10% horse serum (Gibco), and seeded at a density of 80,000 cells/cm² into 48-well cluster plates (Costar) coated with poly-L-lysine (0.1 mg/ml, MW 30,000–70,000; Sigma). After 16 hr, cultures were rinsed three times with serum-free MEM/F12 (1:1, v/v; Gibco) and maintained for 7 d in a modified N2 medium (Bottenstein and Sato, 1979) consisting of MEM/F12 (1:1) supplemented with glucose (5 gm/liter), glutamine (220 mg/liter), HEPES (10 mM), transferrin (50 µg/ml), putrescine (10⁻⁴ M), insulin (5 µg/ml), triidothyronine (5 × 10⁻¹⁰ M), sodium selenite (3 × 10⁻⁸ M), progesterone (2 × 10⁻⁸ M), and gentamicin (50 µg/ml) with medium changes every second day. After 1 d *in vitro* (DIV; day of plating = day 0), cultures were treated with aFGF (human recombinant; kindly provided by Dr. T. Maciag), bFGF (bovine brain extract; Collaborative Research), platelet-derived growth factor (PDGF; Sigma), or interleukin-1 (a gift from Glaxo). The treatment was repeated with every medium change.

To test growth factors for neurotrophic effects in the "absence of glia," glial proliferation was inhibited by treating cultures on day 2 of cultivation for 24 hr with ³H-thymidine (4 µCi/ml; specific activity, 45 Ci/mmol; Amersham).

Mesencephalic glial cultures and preparation of conditioned medium. To establish cultures from embryonic mesencephalic glia, glial proliferation in E14.5 mesencephalic cultures was stimulated with aFGF (50 ng/ml) and heparin (2.5 U/ml) (J. Engele and M. C. Bohn, unpublished observations). After 7 d *in vitro* (DIV), the stimulated cells were dislodged enzymatically (0.1% trypsin), replated at a density of 200,000 cells/cm² into poly-L-lysine–coated culture dishes, and cultivated in serum-free N2 medium.

Conditioned medium (CM) was obtained by incubating mesencephalic glial cultures on day 2 after replating with serum-free N2 medium (200 µl/cm²). After 48 hr, the medium was collected, and cells and cell debris were removed by centrifugation at 1000 and 3000 × g, respectively. CM was aliquoted and stored at −80°C.

Immunocytochemistry. For staining with TH antiserum (1:750; Eugene Tech), cultures were fixed for 20 min with 2% cacodylate-buffered (50 mM, pH 7.3) glutaraldehyde and treated for 5 min with 1% sodium borohydride. For staining with antisera against GFAP (1:1000; Accurate); 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP, 1:750; a gift from Dr. F. A. McMorris); vimentin (Boehringer); the 68, 160, and 200 kDa neurofilament proteins (NF; Boehringer); Leucocyte common antigen (LCA = Ox1; 1:300; Serotec); or fibronectin (Rockland), cultures were fixed for 20 min with 4% PBS-buffered (100 mM) paraformaldehyde. In all cases, cells were permeabilized for 30 min with 0.1% saponine and incubated for 48 hr at 4°C with the primary antibody and for another 2 hr at 20°C with a biotinylated secondary antibody (1:400; Vector). Labeling was detected using the Vectastain ABC kit (Vector Labs) and 3-3′-diaminobenzidine as a chromagen.

Morphometric analysis. The survival of dopaminergic neurons was determined by counting all TH-immunoreactive (TH-IR) neurons per culture well. As an index for neurite length of a TH-IR neuron, the total number of intersections of TH-IR neurites with a reticule grid (1 × 1 cm, subdivided into 100 squares) was determined at a 200× magnification in 10 randomly chosen observation fields (total area, 2.5 mm²) per well and divided by the number of TH-IR somata present in the examined area. GFAP-IR cells were counted in 10 randomly chosen observation fields per well (total area, 2.5 mm²). NF-IR cells were counted in five randomly chosen observation fields per well (total area, 1.25 mm²).

High-affinity dopamine uptake. Cultures were incubated at 37°C with 5 × 10⁻⁸ M ³H-dopamine (specific activity, 44–49 Ci/mmol; Amersham) in the presence of ascorbic acid (100 µM), fluoxetine (1 µM; Lilly), and desipramine (1 µM; Sigma). After 30 min, cultures were rinsed three times, and the radioactivity associated with the cells was determined in an SDS lysate by liquid scintillation counting. Nonspecific ³H-dopamine accumulation was determined in the presence of a 1000-fold excess of unlabeled transmitter. The dopamine-sensitive part of the uptake was considered as specific ³H-dopamine uptake and divided by the number of TH-IR neurons determined in sister cultures (Engele et al., 1989; Reisert et al., 1989).

Autoradiography. Cultures were treated with ³H-thymidine (1 µCi/

ml) at 48 and 24 hr prior to fixation. Cultures stained for GFAP-IR were exposed at 4°C to Kodak NTB2 photographic emulsion and developed after 7 d.

Statistics. Unless stated otherwise, the data are the average from four to six cultures ± SD. Data were submitted to a one-way analysis of variance (ANOVA), and the significance of intergroup differences was determined by applying Student's *t* test. Differences were considered significant at $p \leq 0.05$.

Results

### Effect of growth factors on survival and differentiation of dopaminergic neurons

The number of TH-IR neurons in low-density mesencephalic cultures, maintained for 8 d under previously established serum-free culture conditions (Engele et al., 1991), increased 1.9–2.1-fold after treatment with aFGF or bFGF (Figs. 1, 2). These effects were dose dependent and required half-maximal concentrations of about 3 ng/ml of bFGF and 5 ng/ml of aFGF. Treatment of the cultures with a combination of aFGF and bFGF provided no additive effects on dopaminergic cell survival (Table 1). Previous experiments have shown that heparin potentiates the biological effects of aFGF (Neufeld et al., 1987). However, addition of heparin with aFGF to these low-density cultures caused the cells to form aggregates, and it was not possible to count the number of surviving TH-IR neurons under this condition. Dopaminergic cell survival was also not affected by PDGF or interleukin-1 (Fig. 1).

The effects of these growth factors on the differentiation of dopaminergic neurons were determined by estimating neurite length and high-affinity dopamine uptake after treatment with concentrations of aFGF and bFGF having maximal effects on dopaminergic cell survival (Fig. 3). A small, 1.6-fold increase in neurite length in the presence of aFGF or bFGF was not statistically different from controls. ³H-dopamine uptake per TH-IR neuron was about two times higher in cultures maintained in the presence of bFGF, whereas in aFGF-treated cultures, ³H-dopamine uptake was not statistically different from control levels. Neither PDGF (8 ng/ml) nor interleukin-1 (2 ng/ml) affected neurite length or ³H-dopamine uptake.

Effect of growth factors on mesencephalic glia

To study the effects of growth factors on astrocytes, cultures were immunocytochemically stained for the astroglial marker GFAP. The number of GFAP-IR cells increased four to five times in cultures maintained in the presence of bFGF (10 ng/ml) or aFGF (50 ng/ml) (Table 2). PDGF (8 ng/ml) and interleukin-1 (2 ng/ml) had no effect on the number of GFAP-IR cells in culture. In cultures maintained in the presence and absence of growth factors, GFAP-IR was expressed by two distinct populations of glial cells. The majority of cells was characterized by a stellate morphology similar to type-2 astrocytes. Another smaller population possessed one to two long processes and resembled radial glia (Fig. 4*A,B*). Only a few flat, type-1–like astrocytes were present, and these never displayed GFAP immunoreactivity.

To determine whether the increase in number of GFAP-IR cells was due to glial proliferation or to an improved survival, proliferating cells were labeled with ³H-thymidine (1 µCi/ml for 48 hr). Autoradiograms of these cultures showed that the labeling index of GFAP-IR astrocytes for ³H-thymidine was 6–10 times higher in cultures treated with aFGF or bFGF compared to controls (Table 2, Fig. 4). In addition, both growth factors significantly increased the percentage of non–GFAP-IR cells exhibiting silver grains over their nuclei, with the greatest
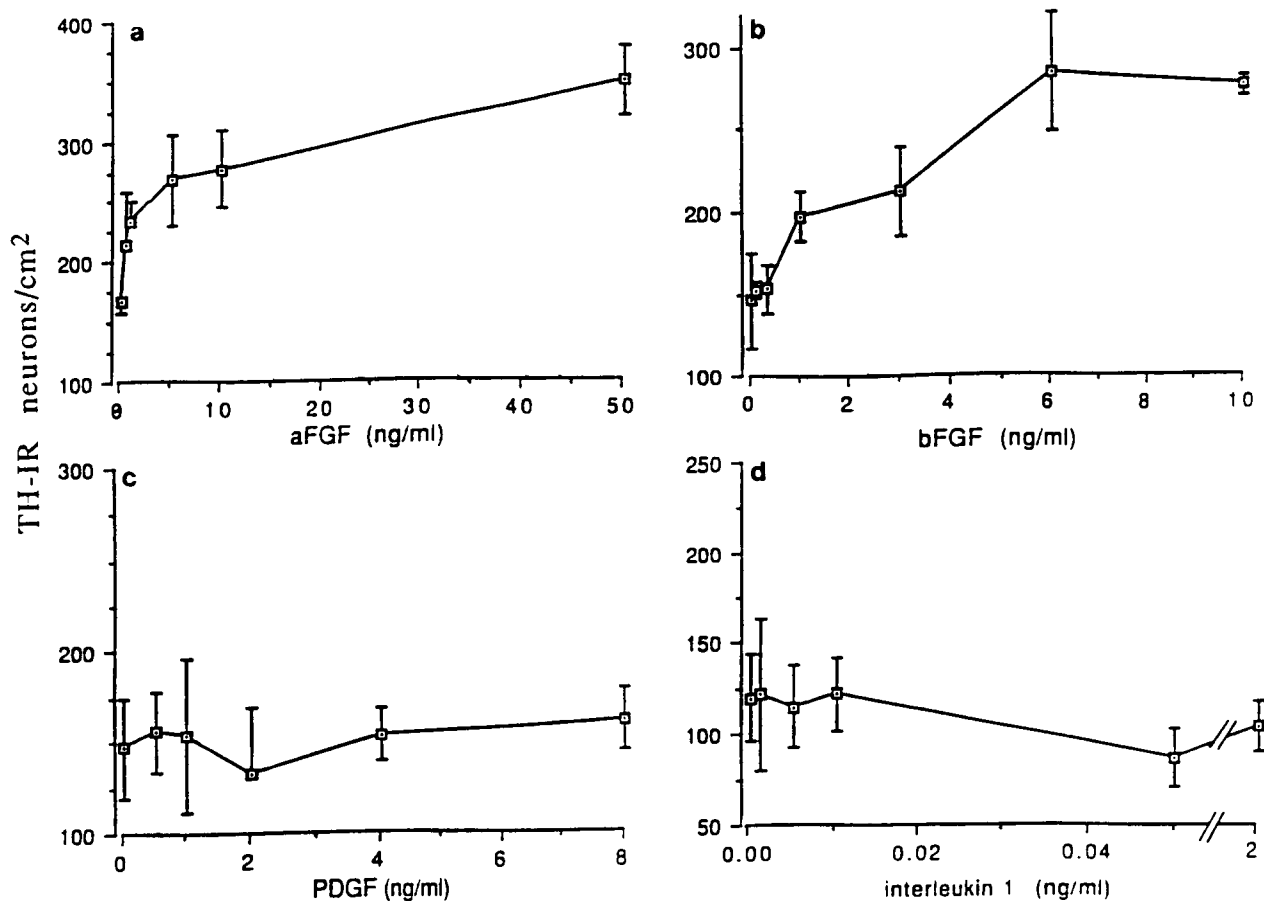
Figure 1. Dose–response effects of aFGF (a), bFGF (b), PDGF (c), and interleukin-1 (d) on survival of dopaminergic neurons. E14.5 mesencephalic cells were seeded at a density of 80,000 cells/cm² and cultivated in serum-free N2 medium and varying concentrations of purified growth factors. After 8 DIV, cultures were fixed and dopaminergic neurons were identified by TH immunocytochemistry. Values are expressed as the mean ± SD (n = 4–6). Significant increases in the number of surviving TH-IR neurons were observed in the presence of aFGF (F = 15.8, df 23, $p \leq 0.0001$) and bFGF (F = 23.5, dF 27, p ≤ 0.0001).

increase elicited by bFGF. In all cases, about one-half of the non–GFAP-IR cells were characterized by an epitheloid morphology, resembling type-1 astrocytes, while the other half expressed a fibrous morphology similar to type-2 astrocytes.

Neurotrophic effects of aFGF and bFGF in cultures with inhibited glial proliferation

To reveal the role of mesencephalic glia in the neurotrophic effects of aFGF and bFGF on dopaminergic neurons, both growth

Table 1. Effect of combined treatments with aFGF and bFGF on dopaminergic cell survival

Treatment	TH-IR neurons/cm²
Control	108 ± 11
aFGF (50 ng/ml)	178 ± 9[a]
bFGF (6 ng/ml)	268 ± 9[b]
aFGF (50 ng/ml) + bFGF (6 ng/ml)	241 ± 40[a]

For description see Figure 1. Combined treatment with growth factors did not significantly increase the number of TH-IR neurons when compared to individual treatments.

[a] $p \leq 0.001$, treatment versus control.

[b] $p \leq 0.01$, treatment versus control.

factors were tested for their survival-promoting effects in mesencephalic cultures with inhibited glial proliferation. Previous attempts to inhibit glial proliferation in serum-free mesencephalic cultures plated at low cell density with cytosine arabinoside (ARA-C) or arabine arabinoside (ARA-A) showed that both substances are neurotoxic under these conditions (Engele et al., 1991). Therefore, we used ³H-thymidine as a mitotic inhibitor for cultured glia. As a result of the short-range radiation of β-particles, it is expected that at a certain concentration of ³H-thymidine the toxicity of the treatment is fairly low on nonproliferating cells but that it is high on proliferating cells that actively accumulate the isotope in their nuclei (Feinendegen, 1967). Treatment of 2-d-old mesencephalic cultures with ³H-thymidine at a concentration of 4 μCi/ml totally abolished the aFGF- and bFGF-induced increase in the number of GFAP-IR astrocytes (Fig. 5A). Moreover, the treatment had no effect on the number of surviving TH-IR neurons in control cultures (Fig. 5B), suggesting that ³H-thymidine concentration does not interfere with the survival of cultured dopaminergic neurons per se. When cultures were treated with 10 μCi/ml of ³H-thymidine, a substantial (60%) decrease in the number of surviving TH-IR neurons was observed. Concentrations of ³H-thymidine of 1 and 2 μCi/ml did not prevent the growth factor–induced glial proliferation. Similarly, treatment of mesencephalic cultures with
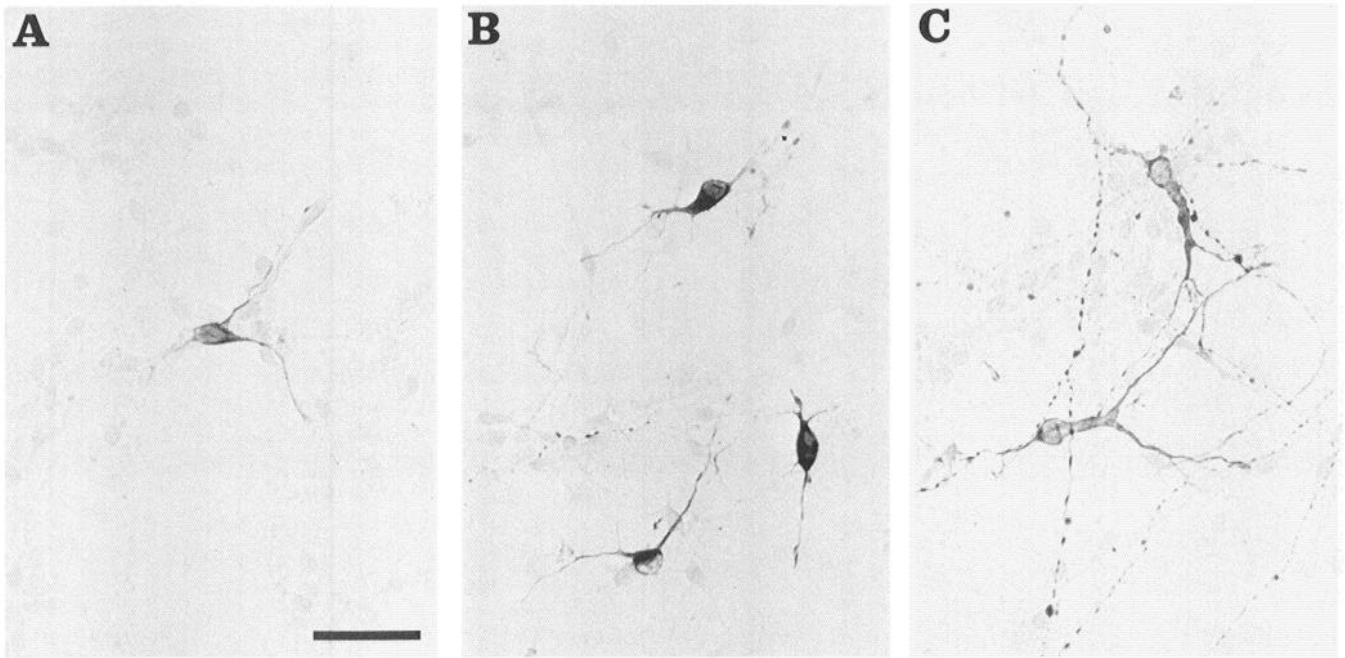
Figure 2. TH-IR neurons in 8-d-old cultures of the E14.5 mesencephalon. *A,* Control; *B,* aFGF (50 ng/ml); *C,* glial CM (60%). Scale bar, 50 μm.

4 μCi/ml of the isotope did not affect the total number of NF-IR neurons (Table 3).

Addition of aFGF and bFGF to cultures in which glial proliferation was inhibited with 4 μCi/ml of ³H-thymidine failed to support dopaminergic cell survival (Fig. 5*B*), suggesting that the survival-promoting effects of both growth factors are mediated by resident mesencephalic glia.

Effect of medium conditioned by mesencephalic glia on dopaminergic cell survival

To determine whether mesencephalic glia support dopaminergic cell survival by a releasable factor, medium conditioned by embryonic mesencephalic glia was tested for neurotrophic effects on dopaminergic neurons. In low-density mesencephalic cultures maintained in the presence of aFGF (50 ng/ml) and heparin (2.5 U/ml), proliferating cells had completely overgrown the culture dish after 7 DIV. Cultures obtained after replating the aFGF and heparin-stimulated cells contained less than 1% NF-IR neurons and were free of fibronectin-IR fibroblasts and LCA (Ox1)-IR microglial cells (Table 4). About 63%

of the replated cells expressed vimentin-IR, a marker for glial cells (Schnitzer et al., 1981). Moreover, 35% of the cells were immunoreactive for the astrocytic marker GFAP and had a stellate morphology (Fig. 6*A*), and about 15% of the cells expressed the oligodendroglial marker CNP and possessed a differentiated oligodendroglia-like morphology (Fig. 6*B*).

When CM derived from aFGF and heparin-stimulated mesencephalic glia after replating was included in low-density E14.5 mesencephalic cultures, the survival of TH-IR neurons increased in a dose-dependent manner (Figs. 2*C*, 7). This increase was 2.2-fold with 60% glial CM. Moreover, glial CM induced a 3 times greater outgrowth of TH-IR neurites (35.0 ± 3.2 intersections/TH-IR neuron with glial CM vs. 11.6 ± 1.4 intersections/TH-IR neuron in controls; $p \leq 0.001$). Importantly, treatment of mesencephalic cultures with glial CM did not result in a general increase in the number of NF-IR neurons (Table 3).

To test whether the survival promoting effect of mesencephalic glial CM on dopaminergic neurons is paralleled by an increase in glial cell number, the glial CM was tested for mitotic

Table 2. Effect of growth factors and glial CM on glial proliferation

	Control	aFGF (50 ng/ml)	bFGF (10 ng/ml)	PDGF (8 ng/ml)	Interleukin-1 (2 ng/ml)	Glial CM (60%)
GFAP-IR cells/cm²	611 ± 141	2205 ± 160[a]	2760 ± 283[a]	605 ± 41 (NS)	643 ± 39 (NS)	641 ± 107 (NS)
GFAP/³H-thymidine (%)	5.1 ± 3.4	50.8 ± 7.6[a]	31.3 ± 3.7[b]	—	—	4.0 ± 2.6 (NS)
Non-GFAP/³H-thymidine (%)	0.9 ± 0.5	6.8 ± 1.7[a]	16.1 ± 0.8[b]	—	—	0.8 ± 0.3 (NS)

E14.5 mesencephalic cultures were initiated as described in Figure 1 and maintained for 8 d in the presence of growth factors. Cultures were treated on days 6 and 7 of cultivation with ³H-thymidine (1 μCi/ml). Cells were fixed and stained for GFAP-IR, and ³H-thymidine labeling was visualized autoradiographically. The number of GFAP-IR cells was determined in 10 randomly chosen observation fields per well and corrected to 1 cm². The numbers of autoradiographically labeled GFAP-IR cells and non–GFAP-IR cells were counted in five observation fields per well and expressed as percentages of the total number of GFAP-IR and non–GFAP-IR cells in the examined area, respectively. Values are expressed as the mean ± SD (*n* = 4). NS, not significant.

[a] $p \leq 0.01$.

[b] $p \leq 0.001$. Treatment versus control.
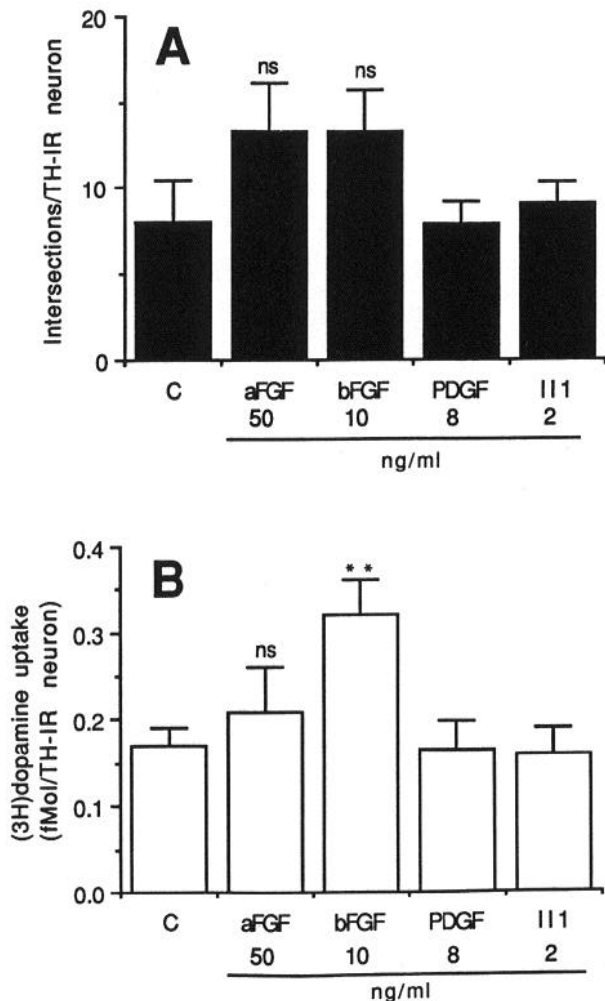
effects on cultured mesencephalic cells. In cultures treated with glial CM, both the number of GFAP-IR astrocytes and the labeling index of GFAP-IR cells and non–GFAP-IR cells for ^{3}H-thymidine (1 μCi/ml) were not significantly different from control cultures (Table 2). Moreover, the glial CM–induced increase in dopaminergic cell survival persisted in cultures in which glial proliferation was inhibited by high concentrations (4 μCi/ml) of ^{3}H-thymidine (Fig. 7). This suggests that the neurotrophic activity contained in the CM acts directly on cultured mesencephalic neurons rather than via glia.

Discussion

In a previous study, we observed that CM derived from several glial lines not only enhance dopaminergic cell survival and differentiation but also stimulate proliferation of mesencephalic glia (Engele et al., 1991). In the present study, we addressed the question whether purified growth factors exert similar dual effects on dopaminergic cell survival and glial proliferation. Exposure of serum-free, low-density cultures of the embryonic

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Figure 3. *A,* Effect of growth factors on neurite-length of dopaminergic neurons. E14.5 mesencephalic cultures were initiated as described in Figure 1 and maintained in the presence of purified growth factors. After 8 DIV, cells were fixed and stained with TH antiserum. The number of intersections of TH-IR neurites with a reticule grid (1 × 1 cm, subdivided into 100 squares) was determined in 10 randomly chosen observation fields per well and was divided by the number of TH-IR somata present in the examined area. Values are expressed as the mean ± SD ($n = 3$). *B,* Effects of growth factors on ^{3}H-dopamine uptake. E14.5 mesencephalic cells were seeded at 80,000 cells/cm^2 and cultured for 8 d in the presence of the indicated concentrations of growth factors. Specific ^{3}H-dopamine uptake was determined within 30 min as described in Materials and Methods. Values are expressed as the mean ± SD ($n = 12$). **, $p \leq 0.01$. *ns,* not significant.
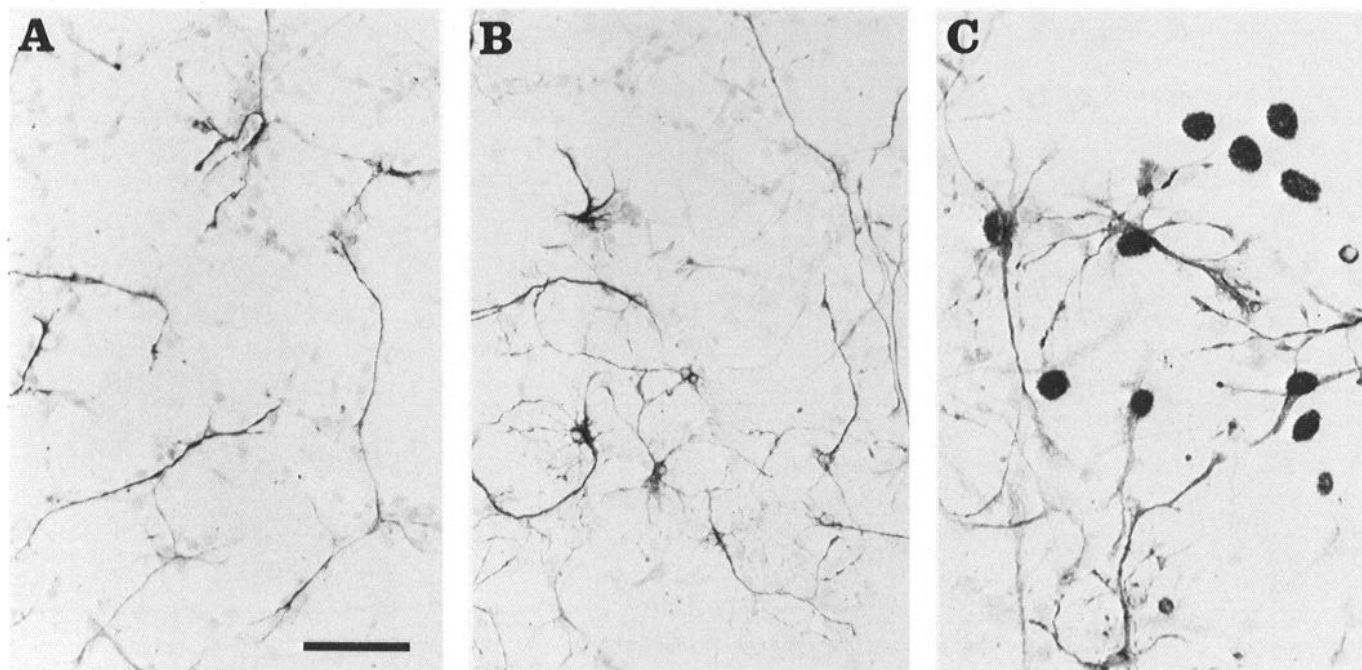
Figure 4. *A,* GFAP-IR astrocytes in 8-d-old mesencephalic cultures maintained in serum-free N2-medium. *B,* GFAP-IR astrocytes in mesencephalic cultures treated with bFGF (10 ng/ml). See text for details. *C,* GFAP-IR astrocytes in bFGF-treated cultures showing autoradiographic labeling for ^{3}H-thymidine. Note that ^{3}H-thymidine labeling is not restricted to GFAP-IR cells. *C,* Scale bar: *A* and *B,* 100 μm; *C,* 50 μm.
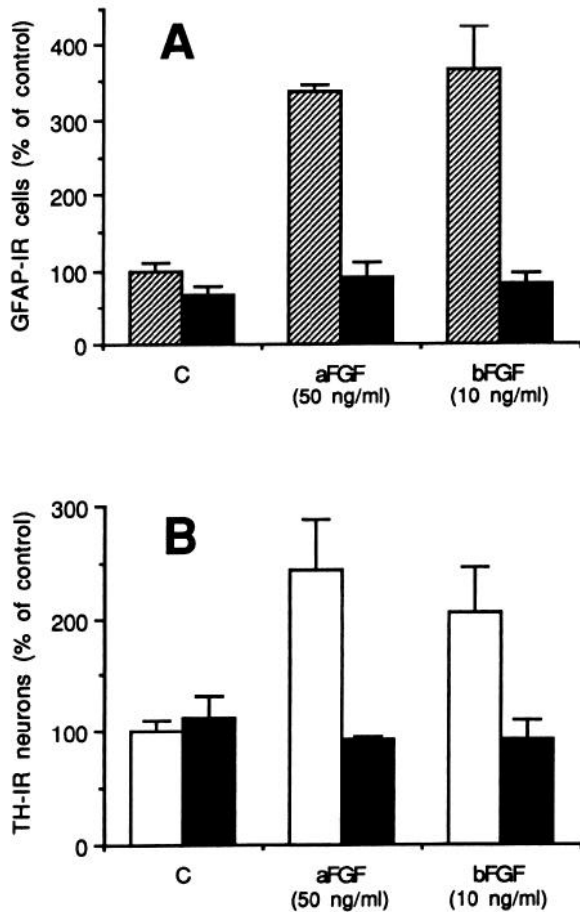
Figure 5. *A,* Effect of aFGF and bFGF on the number of GFAP-IR astrocytes in control cultures (*hatched bars*) and in cultures treated with high concentrations of ³H-thymidine (*solid bars*). Dissociated cell cultures were initiated from the E14.5 mesencephalon as described in Figure 1 and maintained in the presence of growth factors. Cultures were treated on day 2 of cultivation for 24 hr with ³H-thymidine (4 μCi/ml). The number of GFAP-IR astrocytes was determined after 8 DIV as described in Figure 5. *B,* Number of surviving TH-IR neurons in cultures with noninhibited glia proliferation (*open bars*) and in cultures with inhibited glial proliferation (*solid bars*). Cultures were treated as described in *A,* and TH-IR neurons were quantified after 8 DIV. Note that treatment with ³H-thymidine did not affect the survival of TH-IR neurons in control cultures. Values are expressed as mean ± SD (*n* = 4–6).

Figure 6. Morphology of GFAP-IR astrocytes (*A*) and CNP-IR oligodendrocytes (*B*) replated from aFGF and heparin-treated mesencephalic cultures. E14.5 mesencephalic cultures were treated with aFGF (50 ng/ml) and heparin (2.5 U/ml). After 7 d, the stimulated cells were replated and grown another 4–6 d in serum-free N2 medium. Scale bar, 50 μm.

mesencephalon to aFGF and bFGF enhanced dopaminergic cell survival and increased the number of astrocytes. The neurotrophic effect of both growth factors was abolished in cultures with inhibited glial proliferation. Moreover, the survival-promoting effects of aFGF and bFGF on dopaminergic neurons could be mimicked by treating E14.5 mesencephalic mixed neuronal–glial cultures with CM derived from purified mesencephalic glia. Similarly, dopaminergic cell survival was not supported by PDGF or interleukin-1, factors which also did not affect glial proliferation. Taken together, our observations suggest that mesencephalic glia provide a neurotrophic factor(s) for dopaminergic neurons that is distinct from aFGF, bFGF, interleukin-1, or PDGF. Furthermore, the apparent effects of aFGF and bFGF on dopaminergic neurons in these cultures probably occur through increasing the amount of this yet-unknown glial factor by stimulating the proliferation of mesencephalic glial precursors.

The role of FGF as a neuronotrophic factor in the developing brain has been repeatedly proposed. The hypotheses is supported by the recent demonstration of high levels of mRNA coding for aFGF and bFGF, as well as the FGF receptor, in most areas of the embryonic brain (Ernfors et al., 1990; Heuer

Table 3. Effect of glial CM on number of NF-IR neurons

Treatment	NF-IR neurons/cm²
Control	23,920 ± 2623
Glial CM	23,520 ± 763
Glial CM + ³H-Thymidine	22,933 ± 230

E14.5 mesencephalic cultures were established as described in Figure 1 and treated with 60% of glial CM. On day 2, cultures were treated for 24 hr with ³H-thymidine (4 μCi/ml). After 7 DIV, the number of NF-IR neurons was determined in five randomly chosen observation fields per well (total area, 1.25 mm²) and corrected to 1 cm². Values are expressed as mean ± SD (*n* = 3).
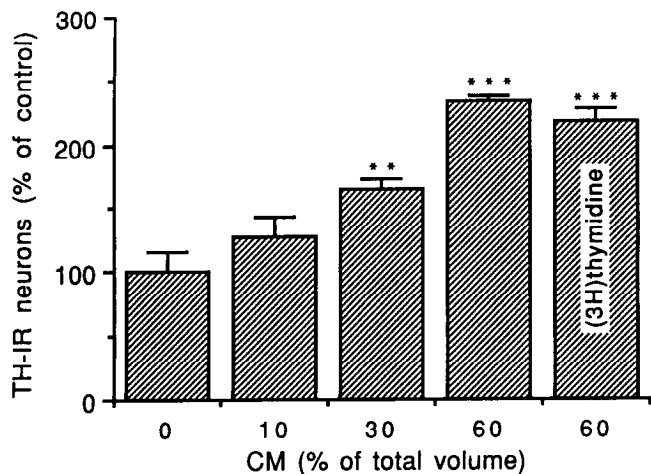
Figure 7. Effect of medium conditioned by aFGF and heparin-stimulated mesencephalic glia on dopaminergic cell survival. CM was obtained by incubating aFGF and heparin-stimulated glia, after replating, for 48 hr with serum-free N2 medium. Effects of various concentrations of glial CM on the number of surviving TH-IR neurons in E14.5 mesencephalic cultures were assessed after 7 DIV. ^{3}H thymidine treatment (4 μCi/ml) was carried out on day 2 of cultivation for 24 hr. Values are expressed as the mean $\pm$ SD (n = 4). **, $p \leq 0.01$; ***, $p \leq 0.001$.

Table 4. Immunocytochemical identification of cells stimulated by aFGF and heparin

Antiserum	Number of immunoreactive cells/1000 plated cells
Anti-vimentin	629 ± 34
Anti-CNP	150 ± 6
Anti-GFAP	337 ± 18
Anti-NF	9 ± 1
Anti-LCA (Ox1)	0
Anti-fibronectin	0

Cells stimulated by aFGF and heparin in E14.5 mesencephalic cultures were replated, grown for 4 d in serum-free N2 medium, and stained with the indicated antisera. Values are expressed as the mean ± SD (n = 4).

et al., 1990; Wilcox and Unnerstall, 1991). Moreover, bFGF and aFGF have been found to support the survival of various CNS neurons *in vitro*, including hypothalamic, cortical, hippocampal, spinal cord, and mesencephalic dopaminergic neurons (Morrison et al., 1986; Walicke and Baird, 1988a,b; Unsicker et al., 1987; Ferrari et al., 1988; Walicke, 1988; Torres-Aleman et al., 1990).

While evidence exists that FGF acts directly on neurons (Walicke and Baird, 1988a), other studies suggested that FGF has indirect effects on neuronal differentiation (Knusel et al., 1990). Several observations suggest that the neurotrophic effects exerted by aFGF and bFGF on cultured dopaminergic neurons are mediated by glial cells. First, in this study it was shown that the increase in dopaminergic neuronal survival after treatment of serum-free mesencephalic cultures with aFGF or bFGF is accompanied by a fourfold increase in the number of mesencephalic astrocytes. The higher labeling index of astrocytes in all these cultures for ^{3}H-thymidine suggests that this increase resulted from a proliferation of glial precursors. Second, the concentrations of aFGF and bFGF of 5 and 3 ng/ml, respectively, required for half-maximal survival of dopaminergic neurons are similar to the concentrations of both growth factors reported to affect glial proliferation and differentiation in cultures from other brain areas (Morrison et al., 1988; Perraud et al., 1988, 1990; Torres-Aleman et al., 1990). Third, the neurotrophic effects of aFGF and bFGF on mesencephalic dopaminergic neurons are mimicked by treating mesencephalic cultures with CM derived from mesencephalic glia previously stimulated with aFGF and heparin and replated in the virtual absence of neurons. Although the aFGF-induced glial cells were rinsed eight times between replating and collection of the CM, the presence of residual exogenous aFGF in the glial CM cannot totally be ruled out; however, it is unlikely that concentrations sufficient to affect dopaminergic cell survival (>1 ng/ml) remained. This is further supported by the finding that unlike aFGF, which increases both the number of dopaminergic neurons and the number of astrocytes in culture, the neurotrophic

effect of the glial CM is not paralleled by an increase in glial cell number. Moreover, the neurotrophic effects of the glial CM persist in cultures with inhibited glial proliferation. These observations suggest that the neurotrophic activity acts directly on mesencephalic neurons and is due to a substance of unknown identity. Furthermore, the absence of any effect of the glial CM on the number of NF-IR neurons suggests that the factor(s) contained in this CM acts specifically on dopaminergic neurons. However, this does not exclude the possibility that the differentiation of other populations of neurons is affected and that these changes are not detected by counting NF-IR neurons. Finally, the neurotrophic effects of aFGF and bFGF on dopaminergic neurons are abolished when glial proliferation is inhibited with high doses of ^{3}H-thymidine. The absence of any influences of the ^{3}H-thymidine treatment on the number of dopaminergic neurons in control cultures excludes the possibility that the treatment itself interferes with dopaminergic cell survival per se. It has been reported previously that aFGF and bFGF are mitogens for neuronal precursor cells (Gensburger et al., 1987; Murphy et al., 1990). However, we observed that the total number of neurons does not decrease in cultures in which proliferating cells are killed with high concentrations of tritiated thymidine (4 μCi/ml). Moreover, cultivated mesencephalic neurons do not express nuclear ^{3}H-thymidine labeling (using 1 μCi/ ml of the isotope) in a 48 hr chase experiment (Engele and Bohn, unpublished observations). These observations suggest that neuroblasts are not proliferating under our culture conditions and exclude the possibility that ^{3}H-thymidine treatment reduces the number of dopaminergic neurons in aFGF- or bFGF-treated cultures by inhibiting proliferation of dopaminergic precursors.

Previous studies have also shown that aFGF and bFGF additively support the survival of hippocampal neurons (Walicke and Baird, 1988b). However, in our system aFGF and bFGF have no additive effects on dopaminergic cell survival, suggesting that the survival-promoting action of both growth factors involves a similar, glial-mediated mechanism. Our previous experiments showing that survival of dopaminergic neurons is twofold higher in mesencephalic cultures treated with conditioned medium from several glial tumor cell lines than in these experiments (Engele et al., 1991) further suggest that the absence of such additive effects is not limited by the original number of dopaminergic neurons plated.

PDGF and interleukin-1 are potent mitogens for cultured O-2A progenitor cells of the optic tract and cortical astrocytes (Giulian et al., 1988; Richardson et al., 1988). The observation that neither of these growth factors stimulates proliferation or

differentiation of embryonic mesencephalic glia suggests that their growth-promoting action may be region specific and/or age dependent.

The identity of the active substance(s) released from the mesencephalic glia remains unknown; however, it appears to be some substance(s) other than aFGF, bFGF, PDGF, or interleukin-1. One possibility is brain-derived neurotrophic factor (BDNF), which recently has been reported to act on mesencephalic dopaminergic neurons (Hyman et al., 1991; Knüsel et al., 1991). It also remains to be determined whether the neurotrophic support exerted by aFGF and bFGF on dopaminergic neurons results from the increased concentration of a neuroactive substance(s) in culture due to the stimulation of glial proliferation or whether the growth factors induce synthesis and/or release of neuroactive substances. Such regulatory influences on glial cells have recently been shown to be exerted by interleukin-1 and bFGF, which upregulate glial mRNA coding for NGF (Hellendall et al., 1990; Vige and Wise, 1990).

The heterogeneity of cells in primary mesencephalic cultures and in cultures obtained by replating aFGF-stimulated glia did not allow an identification of the cellular source of this neurotrophic activity. It is unlikely, however, that neurons represent this source since their number is extremely low in the replated cells. It can further be excluded that these neurotrophic activities derive from fibroblasts or microglia, cell types not detectable in this culture system. Our previous experiments showing that the neurotrophic effects on dopaminergic neurons exerted by CM derived from several glial tumor cell lines are all paralleled by increases in the number of fibrous astrocytes focus attention on astrocytes as likely to be involved in this neurotrophic interaction. Studies demonstrating that dendritic development of dopaminergic neurons is induced in cocultures with mesencephalic astrocytes also support this hypothesis (Chamak et al., 1987).

In summary, our results suggest that in addition to direct actions on neurons, growth factors can influence neuronal survival and differentiation through glial-mediated processes. The demonstration that growth factors can affect neuronal development indirectly not only emphasizes the complexity of cell–cell interactions during development but also suggests that similar interactions may also play an important role in the maintenance and regeneration of neuronal structures in the adult brain.

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