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Basic fibroblast growth factor supports the survival of cerebral cortical neurons in primary culture

(mitogen/neurotrophic factor/neurite extension/central nervous system development)

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ABSTRACT **Bovine basic fibroblast growth factor (bFGF) is a potent mitogen isolated from bovine pituitary glands and brain. The addition of homogeneous bFGF to primary cultures of rat cerebral cortical neurons markedly enhances cell survival and elaboration of neurites. These effects are dose-dependent, with optimal stimulation occurring at a concentration of 500 pg/ml. Maintenance of survival and neurite outgrowth require the continuous presence of bFGF. Other growth factors, such as thrombin, platelet-derived growth factor, β nerve growth factor, and interleukin 2, have no effect on neuronal survival or process formation. Although the cellular site(s) of bFGF synthesis has not yet been established, these results suggest that bFGF may function as a neurotrophic agent in the central nervous system.**

Survival and the acquisition of a differentiated phenotype are critical events characteristic of neuronal development. The spectrum of factors and conditions responsible for regulating these processes have not been elucidated. However, with the discovery of nerve growth factor (NGF) it became clear that certain aspects of neuronal development may be mediated by diffusible molecules. NGF is the most completely characterized of these factors, but its activity is best characterized for neurons of the sympathetic and sensory ganglia (reviewed in refs. 1–3). Since the action of NGF is restricted to several distinct cell types, it is reasonable to assume that additional factors exist for other classes of neurons.

Many different sources have been examined for the presence of neurotrophic factors (4, 5). However, only limited success has been achieved in purifying these substances to homogeneity (6, 7). In general these proteins exert their effects on peripheral neurons. Information on factors influencing survival or maturation of central nervous system neurons has been quite limited.

Basic fibroblast growth factor (bFGF) is a mitogenic polypeptide originally identified in extracts of bovine pituitary (8, 9). bFGF was first purified to homogeneity from the pituitary (10, 11) and later shown to be identical to a basic mitogen isolated from bovine brain (12, 13). The complete sequence has been determined (14). It induces cell divisions in a variety of cell types of mesodermal and neuroectodermal origin (15). bFGF is also a potent stimulator of angiogenesis *in vivo* (16). In this report, we demonstrate that bFGF promotes the survival and elaboration of neurites in primary cultures of rat cortical neurons, indicating that bFGF may function, in addition to its other activities, as a neurotrophic agent in the central nervous system.

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MATERIALS AND METHODS

Materials. Thrombin was a generous gift from D. Cunningham (University of California, Irvine), platelet-derived growth factor (PDGF) was the generous gift of R. Ross (University of Washington), and interleukin 2 (Amgen, Thousand Oaks, CA) was generously supplied by P. Schmidt (University of California, Irvine). Adult mouse submaxillary (for NGF purification) and bovine pituitary glands were from Pel-Freez. Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (DMEM/F-12; 1:1, vol/vol), penicillin/streptomycin, and fetal bovine serum were from Irvine Scientific. Hydrocortisone, putrescine, insulin, prostaglandin $F_{2\alpha}$, and 1-β-D-arabinofuranosylcytosine (araC) were obtained from Sigma. Antiserum against neuron-specific enolase was obtained from Chemicon (El Segundo, CA), anti-neurofilament protein antiserum was obtained from Boehringer Mannheim. Antiserum against glial fibrillary acidic protein (anti-GFAP) was generously supplied by L. Eng (VA Medical Center, Stanford, CA).

bFGF Purification. bFGF was purified from bovine pituitary glands according to the improved procedure of Böhlen *et al.* (11), with a few modifications. Material eluted with 2 M NaCl from the heparin-Sepharose column exhibited two contaminating bands as judged by $NaDodSO_4$/PAGE. These contaminating proteins were removed by separation on a Pharmacia Mono S column, using fast protein liquid chromatography. The column was developed in 20 mM Hepes (pH 8.3) with a linear gradient of 0.1–1.0 M NaCl. The only peak to exhibit mitogenic activity when assayed on BALB/c 3T3 cells appeared as a single band at 16 kDa on PAGE. Its amino acid composition was consistent with the reported amino acid sequence of pituitary bFGF (14).

Preparation of Primary Neuronal Cultures. The initial suspension of cells used for plating the neuronal cultures was prepared as previously described for glial cultures (17). Cells were plated at 10^6 per 35-mm well in DMEM/F-12 (1:1) supplemented with 10% fetal bovine serum, penicillin/streptomycin, and araC (5 μM). Eighteen hours after plating, the cultures were rinsed twice with serum-free culture medium and transferred to a chemically defined medium (CDM) consisting of DMEM/F-12 supplemented with hydrocortisone (50 nM), putrescine (100 nM), prostaglandin $F_{2\alpha}$ (500 ng/ml), insulin (50 μg/ml), and araC (5 μM). This medium is identical to that developed by Morrison and de Vellis (18) to sustain the growth and differentiation of rat brain astrocytes, except for the inclusion of araC. The time of conversion to

Abbreviations: araC, 1-β-D-arabinofuranosylcytosine; bFGF, basic fibroblast growth factor; CDM, chemically defined medium; GFAP, glial fibrillary acidic protein; NGF, nerve growth factor; PDGF, platelet-derived growth factor.

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CDM was designated as day 0. The medium was changed every 3 days, but araC was only added in the first two medium changes. bFGF was added at the conversion to CDM and at every medium change unless otherwise indicated.

Immunocytochemistry of Cerebral Cortical Neurons. Cultures of cortical neurons were plated and maintained as described above. After 5 days of growth in CDM, the cells were fixed by successive incubation (room temperature) with 3.7% formaldehyde and 3.7% formaldehyde containing 0.2% Triton X-100 (10 min each) and incubation at 4°C with 50% (vol/vol) acetone, 100% acetone, and 50% acetone (30 sec each).

For immunostaining, the cultures were incubated with antiserum, to either neuron-specific enolase, neurofilament protein, or GFAP, at a dilution 1:375 in 100 mM Tris·HCl, pH 7.5/150 mM KCl (Tris/KCl) at room temperature for 56 hr in a humidified atmosphere. The cells were rinsed three times for 10 min with Tris/KCl and incubated with biotin-conjugated goat anti-rabbit IgG (diluted 1:250 in Tris/KCl) in a humidified atmosphere at 4°C for 16–18 hr. Cultures were rinsed as above and incubated at room temperature for 2 hr with a complex of avidin and biotin-conjugated glucose oxidase. After an additional rinse, the cultures were washed once with 50 mM Tris·HCl buffer (pH 7.5) and incubated with tetranitro blue tetrazolium in the dark for 15 min at room temperature.

The stained cultures were rinsed several times with 50 mM Tris·HCl buffer, mounted in buffered glycerol (90% glycerol/10% 100 mM Tris), and sealed with transparent nail polish. Cells were viewed through a Nikon Diaphot-TMD inverted microscope. Kodak Tri-Pan black and white film (ASA 400) and high-contrast paper were used for photographic prints.

RESULTS

The addition of purified bFGF to primary cultures of rat cortical neurons resulted in a significant increase in their survival (Fig. 1*A*). Primary cultures of neurons were established in the presence of 5 μM araC to eliminate the presence of dividing, non-neuronal cell types. Thus, the number of cells remaining per dish was observed to decline with time. Control cultures grown in the absence of bFGF exhibited an immediate decline in cell survival. This trend continued for 2 weeks, at which time only 1–2 cells per mm^2 were observed. In contrast, primary neurons maintained in the presence of bFGF survived for up to 2 months in culture.

Survival of cortical neurons *in vitro* in the presence of bFGF was found to be concentration-dependent (Fig. 2). Optimal survival was observed at a bFGF concentration of 500 pg/ml (30 pM), although 100 pg/ml also promoted a significant increase in neuronal survival. However, at concentrations in excess of 500 pg/ml, the survival of cortical neurons was observed to decline. These concentrations closely paralleled the dose–response relationship observed for bFGF as a mitogen for BALB/c 3T3 cells. Optimal mitogenic activity was observed at 1 ng/ml, with half-maximal stimulation occurring at 60 pg/ml (data not shown). The response to bFGF did not level off, and at concentrations ≥10 ng/ml, survival dropped to levels observed with control cultures. This observation was unexpected but not without precedent (12, 19). The declining potency of bFGF observed at higher concentrations might result from, among other things, an inhibitory compound present as a minor contaminant in the preparation or from the rapid down-regulation of bFGF receptors.

Postnatal cerebral neurons maintained in the presence of bFGF exhibited enhanced outgrowth of neurites in comparison to control cultures (Fig. 3). The accumulation of process-bearing cells was greatly enhanced by the addition of bFGF (Fig. 1*B*). A comparison of Fig. 1 *A* and *B* demonstrates that

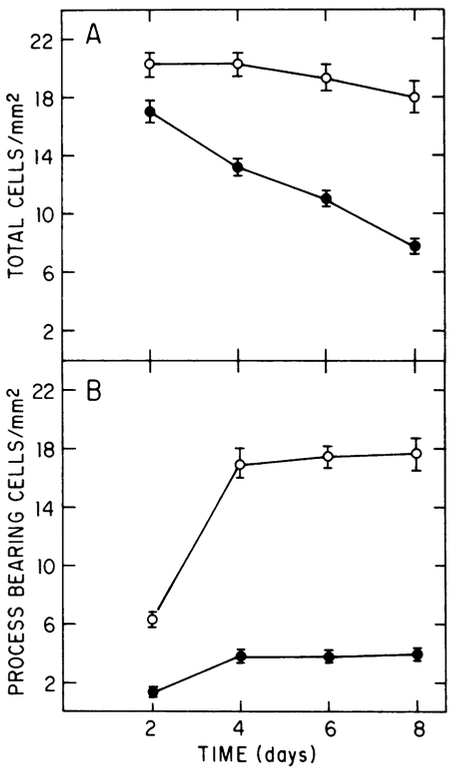

FIG. 1. Survival of primary cortical neurons in the presence of bFGF. Primary cultures were established as described in *Materials and Methods*. Eighteen hours after plating, the cultures were converted to CDM minus bFGF (●) or plus bFGF (1 ng/ml, ○). Either total cells (*A*) or cells bearing processes longer than 100 μm (*B*) were scored in 10 random fields per dish. Three dishes were counted per treatment. The data are expressed as cells per mm^2 (mean ± SEM).

the majority of cells surviving after 8 days in the presence of bFGF have elaborated processes in excess of 100 μm. Neurons surviving in the absence of bFGF exhibited only one or two truncated processes. In contrast, neurons surviving in the presence of bFGF (500 pg/ml) displayed 3–5 distinct neurites with profuse secondary branching. The enhanced

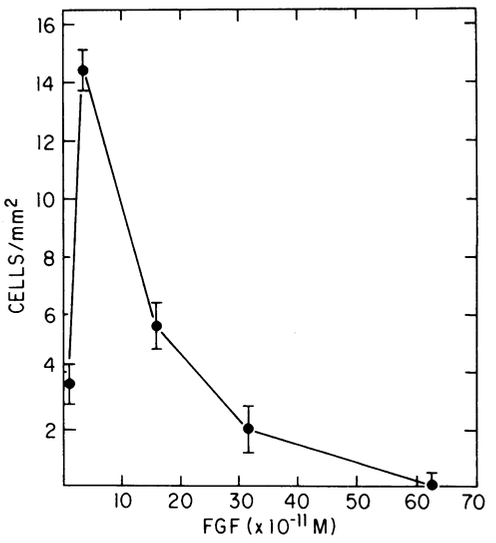

FIG. 2. Neuronal survival as a function of bFGF concentration. Eighteen hours after plating, primary cultures were converted to CDM with various concentrations of bFGF. Five days after conversion to CDM, the cells were counted and the data expressed as described for Fig. 1.

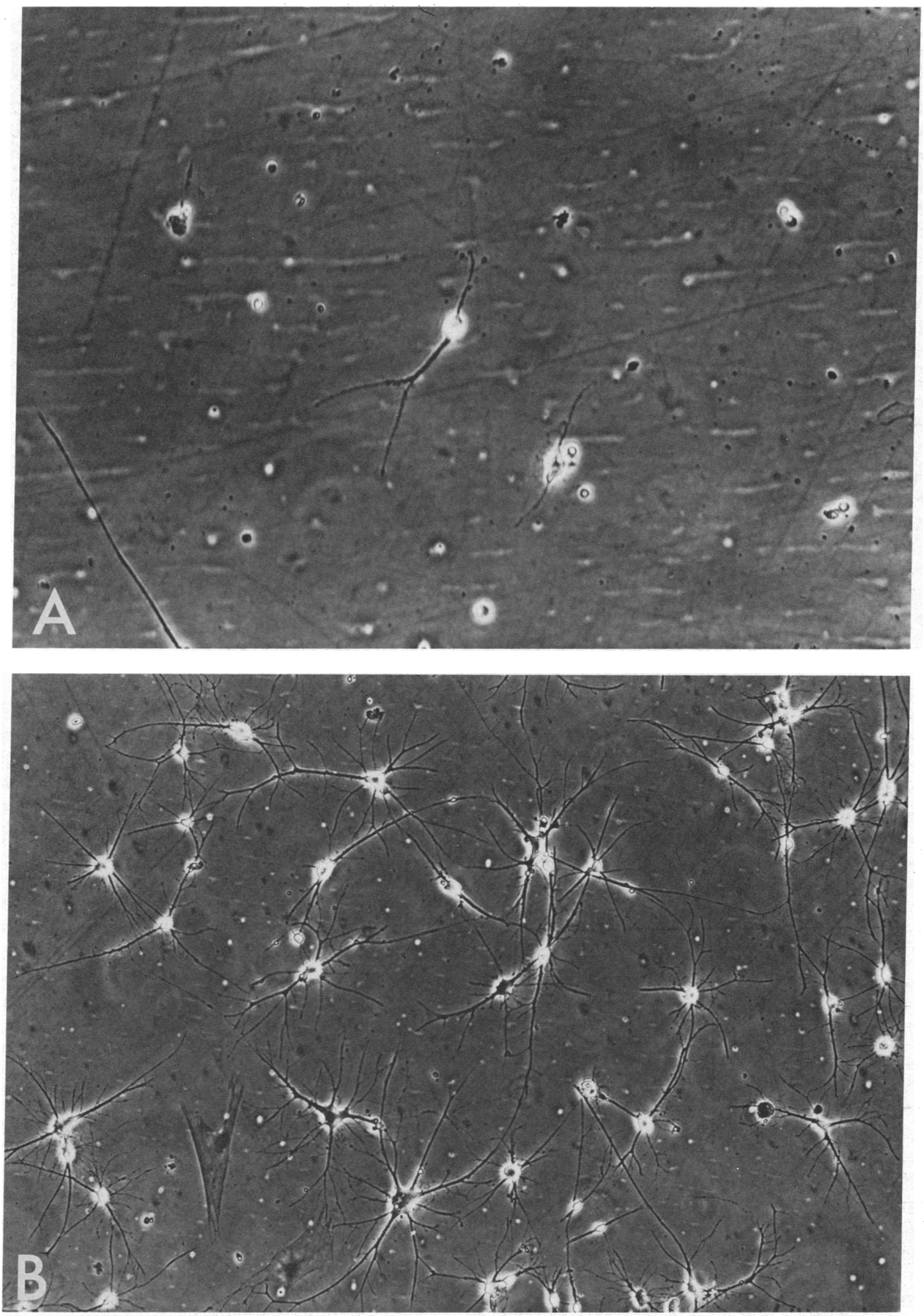

FIG. 3. Phase-contrast micrographs of primary neuronal cultures 10 days after conversion to CDM (*A*) or CDM plus bFGF (500 pg/ml) (*B*). (×225.)

elaboration of processes resulted in neurons making contact with one another through their neurites (Fig. 3*B*).

Cells were identified as neurons on the basis of the expression of neuron-specific enolase (Fig. 4) and neurofilament protein. Approximately 95% of the cells expressed both markers. This was observed in cultures established from postnatal day 2 and embryonic cortex. The number of GFAP-positive cells per culture varied from 3% to 7%.

To determine whether the bFGF response was specific, we assayed other mitogens and growth-promoting substances for neurotrophic activity. Among the other factors tested on cortical neurons were thrombin, β-NGF, PDGF, and interleukin-2. None of these factors had an effect on survival or neurite outgrowth (Table 1).

The mechanism by which bFGF influences survival and neurite outgrowth is not known. Pituitary bFGF presumably acts in a manner similar to that of other polypeptide growth factors via interaction with a cell surface receptor. However, it is not clear whether bFGF receptors exist on neurons or whether the effect is mediated through the small percentage of contaminating glia. To address this question, we transferred neuronal cultures to CDM in the absence of bFGF for various times. The presence of glia was greatest at the initial plating and declined thereafter with the addition of mitotic inhibitors. When bFGF was omitted from the medium for 5 days and then added back to the cells, the number of process-bearing cells was restored to within 80% of the cultures treated since day 0 with bFGF (Table 2). At day 5, GFAP-positive cells comprised about 4% of the total cell population. When bFGF was omitted for 8 days, the surviving cells were still responsive, even though the percentage of glia continued to decline. Although omission for 8 days resulted in a significant loss of total cells, of the cells remaining, 90% responded by elaborating processes more than 100 μm long. The number of GFAP-positive cells at the time of addition equaled 3.0% of the total cell population. Approximately 2 days were required for the elaboration of 100-μm processes, although effects on neurite outgrowth were observed after 24 hr.

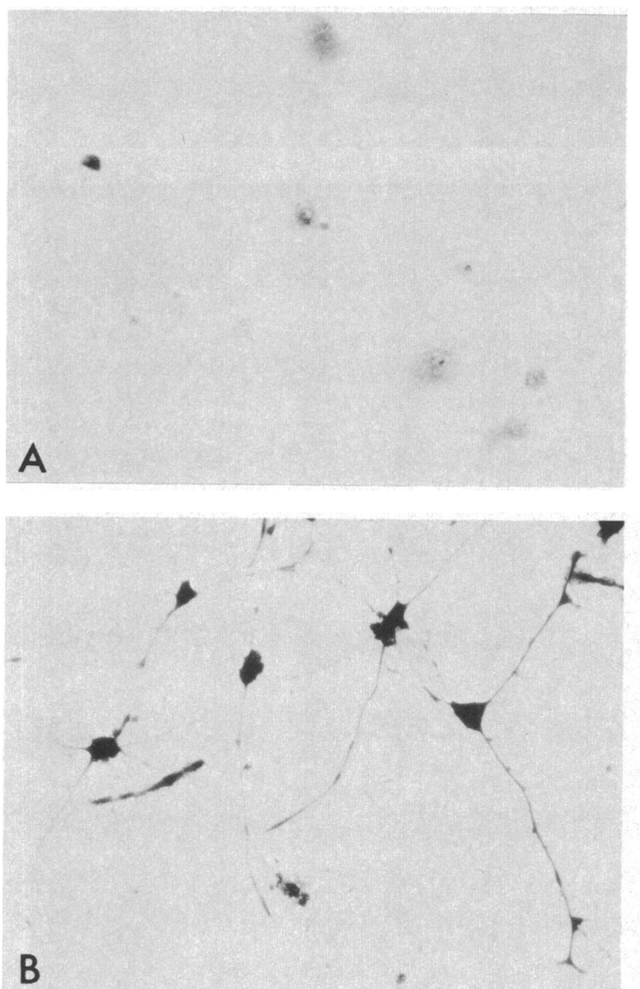

FIG. 4. Immunocytochemical localization of neuron-specific enolase in primary neuronal cultures maintained 5 days in the presence of bFGF. (*A*) Normal rabbit serum (1:100 dilution). (*B*) Rabbit antiserum to neuron-specific enolase (1:100 dilution). (×175.)

Table 1. Influence of growth factors on the survival of process-bearing cells

Factor	Process-bearing cells, no. per mm²
None	3.60 ± 0.32
β-NGF	4.84 ± 0.34
PDGF	4.92 ± 0.32
Thrombin	1.92 ± 0.28
Interleukin 2	2.90 ± 0.16
bFGF	16.90 ± 0.88

Primary cultures were established as described in *Materials and Methods*. On day 0, the cells were converted to CDM plus one of the following: NGF (5 ng/ml); PDGF (10 ng/ml); thrombin (10 ng/ml); interleukin 2 (10 units/ml); bFGF (1 ng/ml). The culture media were changed every 3 days, with fresh factor added at each change. The number of cells bearing processes longer than 100 μm were scored in 10 random fields, using 3 wells per variable. The cells were counted 7 days after conversion to CDM. Data are expressed as mean ± SEM.

DISCUSSION

Neurotrophic activities have been observed in various tissue extracts (20–23) and in culture media conditioned by a variety of different cell types (24–27). However, these activities for neurons of central nervous system origin have, for the most part, not been purified to homogeneity. Thus, their structure, cellular localization, and functional properties remain to be elucidated. Therefore, the demonstration of neurotrophic action by bFGF, a well-defined polypeptide growth factor that influences the survival and maturation of central nervous system neurons, is noteworthy.

bFGF presumably initiates its action on neurons by first interacting with a cell surface receptor. This has recently

Table 2. Continued responsiveness to bFGF after various periods of omission

Day of bFGF addition	Process-bearing cells, no. per mm²		% GFAP⁺ cells on day of addition
	Day of addition	Day 11	
0	—	18.20 ± 0.88	—
5	2.53 ± 0.27	15.20 ± 0.85	4.3
8	3.56 ± 0.28	11.05 ± 0.70	3.0
No addition	—	1.15 ± 0.21	—

Primary neuronal cultures were plated at 10^6 cells per 35-mm dish in medium supplemented with 10% fetal bovine serum. Eighteen hours later, the cultures were shifted to CDM (day 0). Some of the cultures at this time received bFGF (1 ng/ml). The other cultures went for various intervals without bFGF being added to the culture medium. At days 5 and 8, the cells were counted, and then bFGF was added (1 ng/ml) to the medium. bFGF was replenished every 2–3 days with a fresh medium change. The number of cells exhibiting processes 100 μm or longer were scored in 10 random fields, using 3 wells per variable. (Parallel plates grown under identical conditions were processed for GFAP immunocytochemistry.) Final cell counts were made on day 11. Data are expressed as mean ± SEM.

been demonstrated for bFGF on BHK cells (28). However, since we have not provided direct evidence that the effect of bFGF is mediated directly through neurons, there is a possibility that bFGF stimulates the few glial cells present to produce a trophic factor(s) which in turn elicits enhanced neuronal survival and neurite outgrowth. In experiments in which bFGF was omitted from the culture medium for various lengths of time, the number of contaminating glia continuously declined. After 8 days in the absence of bFGF, only 3% of the total cell population was positive for the glial marker GFAP. Many of these cells were vacuolated and appeared unhealthy. Yet, after addition of bFGF, 90% of the cells (GFAP-negative cells) remaining responded by elaborating processes in excess of 100 μm. Further, the number of cells responding to bFGF did not correlate with the percentage of contaminating glia. Thus, we believe that the effects observed for bFGF result from direct interaction of the hormone with neuronal receptors. Since neurons are postmitotic under these culture conditions and *in vivo*, it will be interesting to see whether these cells express a receptor that is structurally related to the receptor found on proliferating cells.

The neurotrophic action of bFGF is not a property shared by all mitogens or growth-promoting factors. The additional factors tested were unable to support the survival of primary cerebral neurons, although this does not mean that they will be ineffective on all other classes of neurons. With the exception of NGF, none of the other factors has been localized within the central nervous system. However, another growth factor, insulin-like growth factor II (IGF-II), does exhibit neurotrophic activity. Recent reports have demonstrated a neurite-promoting effect of this factor (29, 30), as well as the expression of the IGF-II gene in fetal rat brain (31).

It is not clear whether the neurotrophic effect of bFGF is restricted to central nervous system neurons. Cells cultured from the anterior pituitary release into the medium material that crossreacts with antibodies raised against bFGF (32). If bFGF is also released from the pituitary gland *in vivo*, neurons located in the peripheral nervous system could be potential targets. Furthermore, additional responsive targets for bFGF may exist within the central nervous system. bFGF has pronounced effects on central nervous system astrocytes, stimulating their proliferation and inducing the synthesis of GFAP (18, 33, 34). If bFGF is localized in neurons, as is brain acidic FGF (Seroogy, K. G., Fallon, J. H., R.S.M., R.A.B., Gimenez-Guillego, G. and Thomas, K. A., unpublished observations), and functions as a neurotrophic factor, it could well influence these glial properties during the course of normal development or in response to a specific pathogenic event.

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