Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 3012–3016, May 1986
Neurobiology

Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension

(trophic factors/tissue culture)

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Contributed by W. M. Cowan, December 16, 1985

ABSTRACT **Basic fibroblast growth factor (FGF) has been found to increase neuronal survival and neurite extension in a highly purified population of fetal rat hippocampal neurons under well-defined serum-free cell culture conditions. In the presence of FGF, neuronal survival after 7 days in culture on a simple plastic substrate is increased 4-fold, to 54% of the initial population. Survival is increased 2-fold to 40% on polyornithine-laminin. When FGF was bound to plastic or heparin substrates, neurite outgrowth was significantly increased to lengths comparable to those seen with laminin; however, FGF produced no further increase in neurite outgrowth on laminin. Half-maximal survival was observed at FGF concentrations of about 15 pg/ml (1 pM); half-maximal process outgrowth occurred at about 375 pg/ml (20 pM). The responsive cells were identified as neurons by their labeling with tetanus toxin and by antibodies to neurofilaments and to the neuron-specific enolase. Astrocytes, identified by the presence of glial fibrillary acidic protein, constituted about 10% of cells present at 1 week both in the presence and in the absence of FGF. These results strongly suggest that, in addition to its known mitogenic effects on nonneuronal cells, FGF possesses neurotrophic activity for hippocampal neurons.**

The discovery that during normal development substantial numbers of nerve cells in most regions of the central and peripheral nervous systems die and that the proportion of neurons that survive in any given region is closely related to the size of their target field has led to the hypothesis that all nerve cells are dependent for their growth and maintenance on the availability of specific trophic (growth) factors (1–3). The first neurotrophic factor to be identified was "nerve growth factor" (NGF), and the critical role it plays in supporting the survival and growth of sympathetic and certain sensory neurons has been well documented (4, 5). More recently, several putative trophic factors directed toward parasympathetic neurons, spinal cord motoneurons, and various sensory neurons have been isolated and partially characterized (6–8). While it is generally thought that most neurons in the central nervous system (CNS) are similarly dependent on trophic factors, to date much of the evidence for this view is indirect, being based, for the most part, on a number of similarities in the behavior of central and peripheral neurons. Progress in the identification of trophic factors in the CNS has been relatively slow largely because of the difficulty of setting up appropriate assay systems. Most *in vivo* preparations have proved to be both laborious and time consuming and are hardly suitable for screening large numbers of tissue fractions or even purified materials. On the other hand, *in vitro* assay systems generally require the use of primary cell cultures, for which the optimal culture conditions are usually difficult to establish in the absence of appropriate growth factors.

Until recently very few systems have been described in which it is possible to reproducibly obtain adequate cultures of virtually pure neuronal (as opposed to mixed neuronal and glial) cultures. One such system involves the use of dissociated hippocampal neurons from late fetal rats (9–12). The main advantage of this system is that the cell population is unusually homogeneous, the overwhelming majority of the cells at embryonic day 18 being pyramidal neurons (9), and with appropriate controls the proportion of nonneuronal cells in the cultures can be kept to less than 10% (10, 11). Previous studies have established that virtually all the neurons degenerate within 72–96 hr if they are maintained at low density and in a chemically defined medium (10–12), but they can be maintained for periods of several weeks in the presence of explants of hippocampal tissue or a feeder layer of astrocytes, or in medium conditioned by astrocytes (9, 10, 12). We have used this system to examine the effects of highly purified basic fibroblast growth factor (FGF) on the survival of the isolated neurons and on their capacity to extend neurites. Since FGF has been isolated from brain (13), it seems likely that it may be involved in similar processes *in vivo*.

MATERIALS AND METHODS

FGF. Basic FGF was purified from bovine pituitary by heparin-Sepharose affinity chromatography; from reverse-phase high performance liquid chromatography the preparation is estimated to be greater than 90% homogeneous (13). Its structural characterization and sequence have been published elsewhere (14, 15).

Tissue Culture Methods. Hippocampi were dissected from embryonic day 18 Sprague–Dawley rats and incubated for 15 min in 0.25% trypsin. The solution was removed and residual trypsin was inhibited with 60 μM phenylmethylsulfonyl fluoride, while the cells were dissociated by trituration through a silane-treated pipette. The dissociated neurons were plated into 96-well plates (Costar 3596) at a density of 5000 neurons per 100 μl of medium in each 6-mm well (i.e., about 16,000 neurons per cm^2). The neurons were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, penicillin at 100 units/ml, streptomycin at 100 units/ml, Fungizone at 0.25 μg/ml, 20 mM KCl, catalase at 2.5 μg/ml (16), superoxide dismutase at 2.5 μg/ml, and the N2 supplements of Bottenstein and Sato (17). The cells were grown either directly on tissue culture plastic or on previously prepared substrates of laminin or heparin. Laminin substrates were prepared by incubation over polyornithine-coated plastic in the manner described by Manthorpe *et al.* (18) with laminin obtained from Bethesda Research Labora-

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Abbreviations: CNS, central nervous system; FGF, fibroblast growth factor; NGF, nerve growth factor.

tories. Heparin substrates were prepared by incubating a 10 μg/ml solution (Sigma H3125) overnight on previously applied polyornithine.

Determination of Neuronal Survival and Neurite Outgrowth. At appropriate times, the cells were fixed in phosphate-buffered 2% glutaraldehyde. Survival was quantified by counting, under phase-contrast optics, all the identifiable neuronal profiles in a series of transverse strips covering about 10% of the culture area. Concurrent immunohistochemical studies with tetanus toxin and antibodies to neurofilaments and neuron-specific enolase facilitated the recognition of neurons versus nonneuronal cells. Neurite outgrowth was estimated with a stylus coupled to a Bausch and Lomb Hipad digitizer, using 8 × 10 photographic enlargements of ×200 phase-contrast micrographs taken from regions of moderate cell density. The fields were selected to contain 25–40 neurons, sufficiently separated to permit accurate measurement of their processes. In each case the first 100 clearly defined neurons were selected and only the longest process of each cell was measured.

Immunohistochemistry. Immunohistochemistry was performed as described by Haan *et al.* (19). The antibody to glial fibrillary acidic protein (GFAP) was generously provided by L. Eng of Stanford University; tetanus toxin and antitoxin, by R. O. Thomson of Wellcome Laboratories; and the anti-enolase antibody, by our colleague B. Boss. All other antibodies were obtained commercially.

RESULTS

FGF Promotes Neuronal Survival

The survival of dissociated hippocampal neurons in serum-free medium on two different substrates, plastic and laminin, is summarized in Figs. 1 and 2. As reported previously for similarly prepared cultures in other laboratories, we have found, using a variety of standard immunocytochemical markers, that at least 90% of the surviving cells are neurons (10, 11). On laminin neuronal survival is excellent at 1 day (Fig. 1*B*); this probably reflects the combined effects of moderately high cell density (5000 cells per 6-mm well) and the presence of pyruvate and catalase in the medium (9, 11, 16, 20). Plastic (Fig. 1*A*) is a somewhat less efficient substrate and, on average, only about 60–80% of the cells seeded survived for 24 hr. The neurons did not adhere well to plastic if they were exposed to serum or bovine serum albumin after the dissociation or in the culture medium.

During the first few days in culture, the neurons grow rapidly and elaborate neurites. By the 4th or 5th day, the cell bodies and processes appear granular under phase-contrast optics and are evidently degenerating. By the 7th day only about 10–20% of the neurons remain (Figs. 1, and 2 *A* and *C*), and in some experiments as few as 5%. The overall survival of neurons on lamin and plastic is more or less comparable at 24 hr, but thereafter the number of neurons surviving on plastic is usually

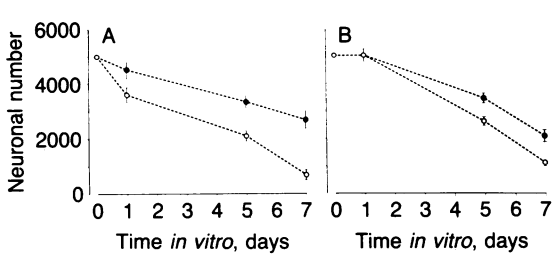

FIG. 1. Survival of hippocampal neurons grown on plastic (*A*) or polyornithine/laminin (*B*), under control conditions (○) or in the presence of basic FGF at 300 pg/ml (●). Values are mean ± SEM for determinations on six duplicate cultures.

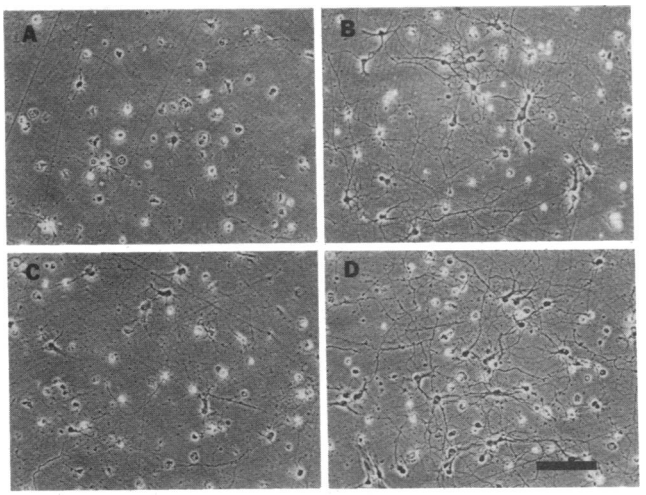

FIG. 2. Survival of hippocampal neurons in the presence of FGF. Photographs were taken of representative phase-contrast microscopic fields from 7-day-old cultures used to generate Fig. 1. Cultures in *A* and *B* on plastic substrate; *C* and *D* on polyornithine-laminin. *A* and *C* illustrate control conditions; *B* and *D*, in the presence of basic FGF at 300 pg/ml. Bar = 100 μm.

lower (Figs. 1*A* and 2*A*). Similar modest increases in the survival of highly purified neuronal populations on laminin substrates have been observed by others (18, 21, 22).

The addition of basic FGF (300 pg/ml, 20 pM) to the culture medium significantly increases the survival of the dissociated hippocampal neurons at both 5 and 7 days. On laminin (Figs. 1*B* and 2*D*), neuronal survival is increased nearly 2-fold, to about 40% of the number of cells present at 24 hr; on plastic (Figs. 1*A* and 2*B*) survival is even better, with a nearly 4-fold increase to almost 60% of the population at 24 hr. The greater survival on plastic reflects, to some extent, the initially smaller number of surviving neurons, but it is clear that in the presence of FGF neurons survive as well on plastic as on laminin. In fact, the actual number of surviving cells is frequently greater on plastic, as in the experiment illustrated in Fig. 1. On both substrates enhanced long-term survival is accompanied by a more elaborate network of neuronal processes (Fig. 2 *B* and *D*).

The population of neurons supported by FGF at 7 days continues to die slowly, so that there are few survivors by 14 days *in vitro*. In the experiment illustrated in Figs. 1 and 2, FGF was added to the medium only at the beginning of the experiment. More frequent additions of FGF to the medium do not seem to improve neuronal survival, nor does the addition of hydrocortisone and triiodothyronine (10) or the use of a more enriched serum-free medium (23) (data not shown).

The minimal concentration of FGF needed for neuronal survival was determined by counting the number of neurons present after 5 days *in vitro* in serial 2-fold dilutions of the factor. In the experiments shown in Fig. 3, half-maximal survival was observed at about 15 pg/ml on plastic, and 20 pg/ml (1 pM) on laminin. From a series of such determinations the minimal concentration would appear to be in the range of 10–30 pg/ml. These figures compare favorably with the half-maximal concentration of 30–50 pg/ml for inducing mitosis in endothelial cells with the same preparation of FGF (13, 14). Conversely, there was no evidence that FGF is toxic even at concentrations as high as 300 ng/ml (data not shown).

Since it is known that hippocampal neuronal survival is enhanced in the presence of astrocytes (9, 10, 12) and that even crude preparations of FGF can stimulate astrocytes to multiply and to become more fibrillar and neuron-like in appearance (24–26), it was important to demonstrate that (*i*) the surviving cells were indeed neurons and (*ii*) neuronal

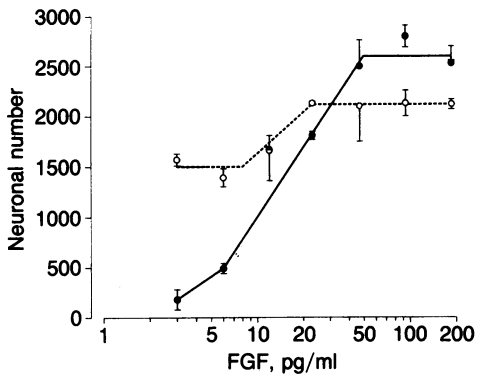

FIG. 3. Determination of FGF concentration necessary for survival on plastic (●) or polyornithine/laminin (○). Four duplicate 5-day-old cultures were examined at each concentration of FGF. Values are mean ± SEM.

survival was not merely enhanced as a result of astrocyte proliferation. For this demonstration week-old sister cultures of those used to generate Figs. 1 and 2 were stained with an antibody to glial fibrillary acidic protein specific for astrocytes. Control cultures on the laminin substrate contained 100 ± 28 astrocytes, while FGF-treated cultures contained 220 ± 32 astrocytes. Although the absolute number of astrocytes was higher in the cultures containing FGF, under both conditions the ratio of neurons to astrocytes was about 10:1. An astrocytic population of less than 10% is comparable to that observed by others in serum-free hippocampal cultures and, by itself, this proportion of astrocytes has been found to be insufficient to support neuronal survival (10, 11).

Both control and FGF-treated cultures were also examined with antibodies against neurofilaments or neuron-specific enolase and with tetanus toxin. In each case the vast majority of the cells and processes were stained with these reagents, confirming that most of the cells in our cultures were, indeed, neurons. In one set of 8-day-old cultures between 9% and 17% of the cells failed to bind tetanus toxin; most of these cells were probably astrocytes, but some could have been immature oligodendrocytes, which have also been reported to divide in the presence of FGF in a similar serum-free medium (27, 28).

FGF Promotes Neurite Outgrowth

The 5- to 7-day-old cultures maintained with FGF showed an appreciably denser network of processes (Fig. 2 *B* and *D*). As this could simply be secondary to improved neuronal survival, we have examined neurite outgrowth in 24-hr cultures, in which survival is not significantly enhanced by FGF. For these experiments, in addition to using plastic and laminin as substrates, we used heparin because it is known to bind FGF avidly (13), and it seemed likely that restricting the availability of FGF to the plane of the substrate might increase the stimulus to neurite elongation. FGF was accordingly bound to the substrates tested during a 3-hr incubation; thereafter, the plates were rinsed with fresh medium, and the test neurons were added. The results of a typical experiment of this kind are shown in Figs. 4 and 5.

On heparin alone, neurite elongation was poor. Nearly 30% of the neurons lacked processes, and more than two-thirds of

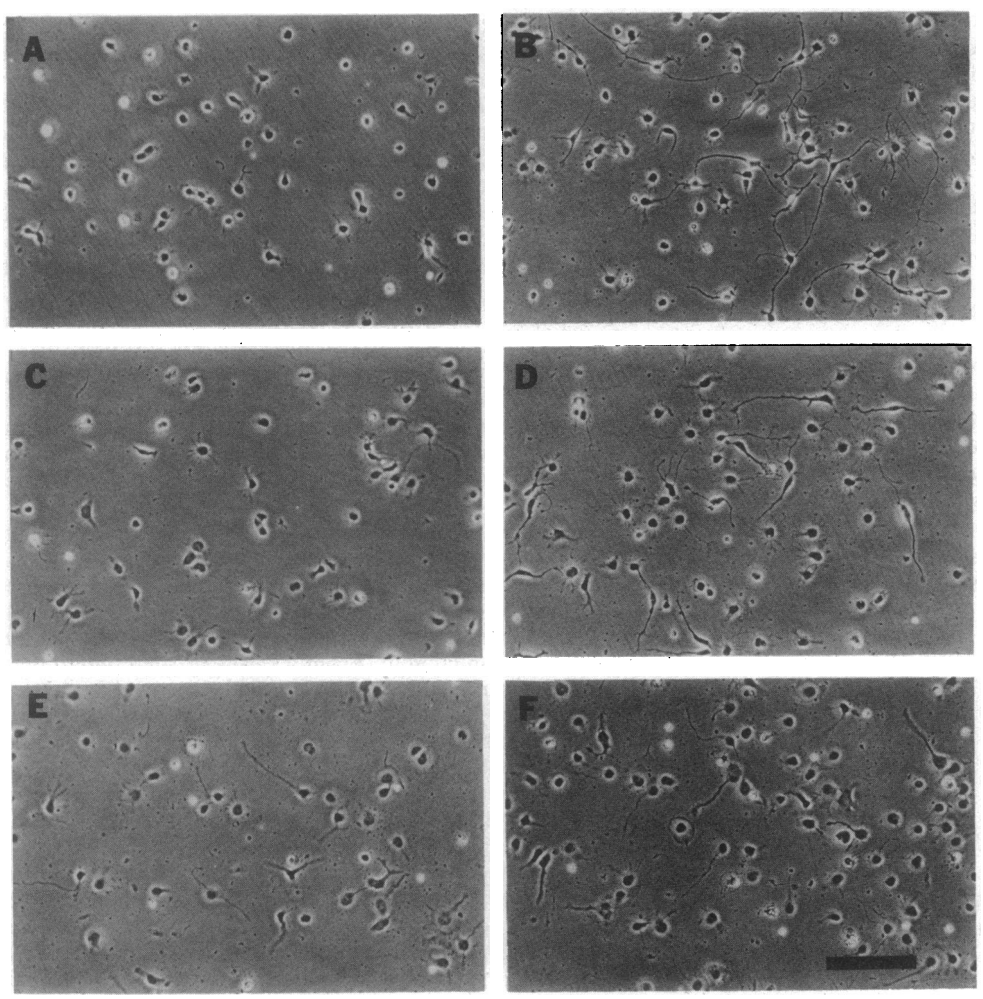

FIG. 4. Effects of FGF on neurite extension in 24-hr cultures of hippocampal neurons. Representative ×200 phase-contrast microscopic fields are from cultures used to generate Fig. 5. Three substrates were compared: polyornithine-heparin (*A* and *B*), tissue culture plastic (*C* and *D*), and polyornithine-laminin (*E* and *F*). Samples *A*, *C*, and *E* were control conditions; samples *B*, *D*, and *F* were after incubation of each substrate with a solution of basic FGF at 3 ng/ml. Bar = 100 μm.

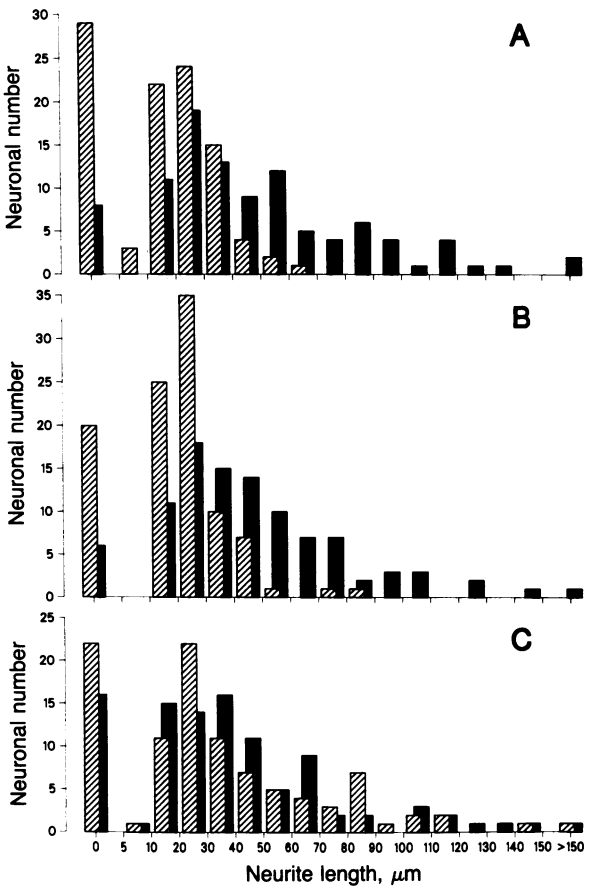

FIG. 5. Histograms representing distributions of neurite lengths in 24-hr cultures of hippocampal neurons grown on polyornithine/heparin (*A*), plastic (*B*), or polyornithine/laminin (*C*). Hatched bars represent control conditions; solid bars represent cultures incubated with 50 μl of basic FGF at 3 ng/ml. The longest neurites were measured on 100 neurons for each condition.

the remaining cells had no processes longer than 30 μm (Fig. 4*A* and hatched bars in Fig. 5*A*). The results on tissue culture plastic were only slightly better (Figs. 4*C* and 5*B*). Even on laminin, about 20% of the cells had no neurites; however, there was a distinct shift in the distribution of neurite lengths among the remaining neurons, with 56% having processes longer than 30 μm (Figs. 4*E* and 5*C*). These observations are consistent with previous findings on neurite outgrowth on these substrates (18, 21, 22, 29–31).

The addition of FGF had a particularly striking effect on neurite elongation on heparin. As shown in Fig. 5*A*, the proportion of cells without processes fell from 29% to 8%, and of the remaining cells 88% had neurites longer than 30 μm, and 10% had neurites that were 100 μm or more long. The presence of such lengthy neurites is striking even on visual inspection (Fig. 4*B*) and the change in the distribution of neurite lengths was highly significant ($P < 0.001$, χ^2). The cells with long processes were identified as neurons in control experiments by their staining with anti-enolase and tetanus toxin. As Fig. 5 shows, the FGF/heparin substrate was actually more effective for neurite outgrowth than was laminin (compare Fig. 5 *A* and *C*). The distribution of neurite lengths on FGF/heparin versus laminin differed at the $P < 0.05$ level, but FGF/laminin and FGF/heparin were not significantly different.

A similar, though less striking, result was obtained with a plastic substrate: the percentage of neurons without processes declined from 20% to 6%, and the proportion of the remaining population with neurites longer than 30 μm increased from 25% to 69% (Figs. 4*D* and 5*B*). By contrast, FGF bound to laminin seemed not to promote neurite outgrowth beyond that observed on laminin alone (compare Figs. 4 *E* and *F* and 5*C*). To exclude the possibility that the FGF had merely failed to bind to the laminin, it was added to the medium in another set of experiments; the results in these cases were not significantly different (data not shown). Since cells perform almost as well on laminin alone as on plastic or heparin in the presence of FGF, it is possible that neurite elongation is already nearly maximally stimulated on laminin; however, we cannot exclude the possibility that the laminin preparation itself contains FGF. Blocking antibodies against FGF could resolve this issue.

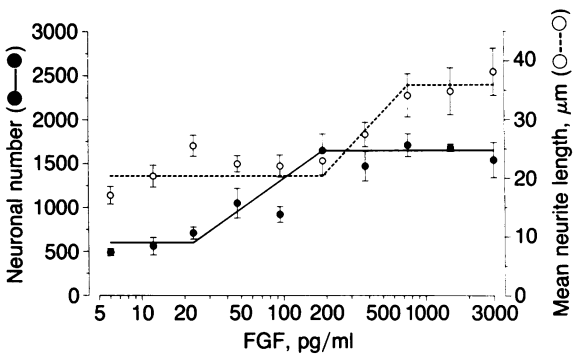

FIG. 6. Determination of FGF concentration necessary for survival versus neurite extension on polyornithine/heparin. Fifty-microliter aliquots of solutions with the indicated concentrations of basic FGF were incubated on the substrate for 3 hr; cells were added after the substrate had been rinsed. The fraction of FGF actually bound was not determined. One set of cultures was fixed at 24 hr and neurite lengths for 100 neurons were determined at each FGF concentration; values are mean ± SEM (○). Another set of cultures was evaluated after 5 days for neuronal survival; values are means ± SEM for six duplicate samples (●).

The concentration of FGF required to promote neurite outgrowth was determined on heparin because of the more striking differences found with this substrate. In the experiment illustrated in Fig. 6, half-maximal stimulation occurred at a concentration of 375 pg/ml (about 20 pg per 6-mm well). In other determinations it was found to range between 200 and 500 pg/ml. Sister cultures were allowed to survive for 5 days, and the remaining neurons were counted to obtain a half-maximal concentration for survival on heparin. This was found to be about 50 pg/ml, a figure which is reasonably similar to that determined for plastic and laminin (Fig. 3). Neurons surviving in the lower concentrations of FGF showed good process outgrowth at 5 days, although no significant enhancement of outgrowth was noted at earlier times.

DISCUSSION

Basic FGF is a 146 amino acid protein with a molecular weight of 16,000 and an isoelectric point of about 9.6. It has previously been recognized as a mitogen for a variety of mesodermal cells, including fibroblasts, endothelial cells, chondrocytes, and adrenal cortical cells (13–15, 32). Our findings indicate that it also has profound effects on the survival and growth of dissociated hippocampal neurons *in vitro*. Thus, in serum-free medium, and on a plain plastic substrate, over 50% of the initially seeded population of neurons can survive for 1 week in the presence of FGF, whereas in its absence less than 15% of the cells survive. Moreover, FGF appears to be extremely potent, only 10–30 pg/ml (about 1 pM) being required for half-maximal survival of hippocampal neurons. This compares favorably with the concentration of nerve growth factor (NGF) required by

sympathetic neurons (about 2 ng/ml, or 100 pM) (5). It is also comparable to the concentration of FGF required to stimulate mitosis in aortic arch endothelial cells (about 30–50 pg/ml) (13, 14). Since basic FGF has been purified from brain extracts (13) it may well be available to hippocampal neurons *in vivo*, and it is reasonable to suppose that it may serve a similar function during normal growth and development.

A possible complicating factor in our experiments is the fact that the hippocampal neuronal cultures are contaminated by about 10% nonneuronal cells. Although the proportion of such cells is unchanged in the presence or absence of FGF, the possibility exists that FGF may act primarily on these nonneuronal cells, and that they, in turn, support neuronal survival. At present we cannot exclude this possibility, but the low density of astrocytes in our cultures would argue against this interpretation. And the marked effects on neurite outgrowth at early times *in vitro* are difficult to attribute to an indirect effect of this kind.

In addition to supporting neuronal survival, FGF is able to promote neurite extension, although generally only at higher concentrations than those required for cell survival. In this respect FGF resembles NGF (4, 5) but differs from certain other trophic factors, such as ciliary neuronotrophic factor (CNTF) (6), which appear to promote cell survival without affecting neurite outgrowth. In our experiments FGF bound to either heparin or plastic induces neurite extension about as well as the extracellular matrix component laminin (18, 22, 29–31). Half-maximal effects of FGF were seen with solutions containing only 375 pg/ml (about 20 pM). Assuming 100% binding, one can calculate that this corresponds to about 0.6 pmol/mm^2; this is indicative of unusually high potency for promoting neurite outgrowth. We have not attempted to measure the minimal concentration of laminin needed for neurite promotion in hippocampal cultures, but others have reported figures around 50 ng/ml (about 40 pM, assuming a molecular weight of about 10^6) (18). Since the binding efficiencies of FGF and laminin are probably different, it would be a mistake to place too much weight on these numerical comparisons, but it is clear that FGF is about as potent in stimulating neurite extension as laminin.

A commercially available preparation of FGF, of unspecified purity, has previously been reported to stimulate neurite extension from PC12 cells (33). This suggests that the neurite-promoting effects of FGF may not be restricted to hippocampal neurons but may be of fairly widespread occurrence. Initial results from examining the range of responsive central and peripheral neurons indicate that at least some neocortical and some striatal neurons also respond to FGF and that our purified FGF preparation is capable of transforming PC12 cells into a characteristic neuronal phenotype at concentrations about 1/100th of that for NGF.

There is also evidence that FGF may be mitogenic for both astrocytes and oligodendrocytes (24–28). Because commercially available FGF was used in most of these studies, it is difficult to compare their findings with our own. We did not observe astrocyte proliferation, but this may well be because the serum-free medium we have used is not permissive for astrocyte growth (25); indeed, we have found that basic FGF may stimulate astrocyte division in the presence of serum. Considerably more work with purified FGF is required before we will know whether basic FGF has significant effects on all three cellular components of the CNS—neurons, astrocytes, and oligodendrocytes. Elucidating how FGF acts among these potential cellular targets could have important implications for our understanding of such phenomena as the widespread occurrence of neuronal death during embryonic development, the reactive gliosis that follows neuronal injury, and even the failure of regeneration in the adult CNS.

We thank B. Boss, J. Fawcett, G. Banker, and D. Schubert for helpful discussions and insights into these experiments, C. Harrison for her excellent technical assistance, and P. Thomas for her secretarial help. This work was supported in part by Grants EY-03653 and NS-16980 from the National Institutes of Health and was carried out while P.W. was in receipt of Teacher Investigator Award I KO7 NS 00948-01 from the National Institute of Neurological and Communicative Disorders and Stroke.

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