Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 961–965, February 1991
Neurobiology

Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3

(choline acetyltransferase/γ-aminobutyratergic neurons/cell culture)

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Communicated by Richard F. Thompson, November 19, 1990

ABSTRACT **Recombinant human brain-derived neurotrophic factor (rhBDNF) and neurotrophin 3 (rhNT-3), two recently cloned molecules closely related to nerve growth factor (NGF), were produced from human cDNA expressed in human embryonic kidney cells. The recombinant proteins were tested in cultures of dissociated fetal rat brain cells containing basal forebrain cholinergic neurons. rhBDNF stimulated the differentiation of the cholinergic neurons, similar to NGF, which is well established as a neurotrophic factor for these cells. However, rhBDNF was particularly effective during the first few days *in vitro*, whereas the stimulatory action of rhNGF was more pronounced later in the development of the cultures. This finding indicates the existence of different time periods of responsiveness of the cholinergic neurons to BDNF and NGF. To assess the selectivity of the effect of rhBDNF on cholinergic neurons, its actions were tested in cultures of ventral mesencephalon containing dopaminergic cells. In contrast to NGF, which does not affect central dopaminergic neurons, rhBDNF increased dopamine uptake activity. The findings suggest that BDNF stimulates survival or differentiation of other cells besides the cholinergic neurons.**

Developmental growth, maintenance of function, and plasticity of neuronal populations are influenced by soluble proteins including neurotrophic, growth, and neuronal differentiation factors. Nerve growth factor (NGF), the first and best characterized neurotrophic factor, promotes survival, growth, and biochemical differentiation of peripheral sympathetic and sensory neurons and basal forebrain cholinergic neurons (1–4). Survival and growth of these cholinergic cells are also affected by factors primarily known to target non-neuronal cells. These factors include fibroblast growth factor, epidermal growth factor, and the insulin-like growth factors (5–7). Based on work by Barde *et al.* (8) brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3), two neurotrophic factors closely related to NGF, were discovered and cloned (9–12). Both molecules share ≈50% of their amino acids with NGF. BDNF, NT-3, and NGF support survival of selected populations of chick sensory neurons, suggesting independent roles in the regulation of neuronal survival during development. BDNF was originally purified from adult pig brain, and high levels of BDNF and NT-3 mRNA occur in adult rodent brain (9–12), suggesting an important role of BDNF and NT-3 in the central nervous system. Identification of neuronal populations responsive to BDNF and NT-3 is an important initial step in elucidating their brain function. We studied the response of the basal forebrain cholinergic neurons, a neuronal population important for cognitive brain functions, and of other cells, including dopaminergic neurons of the mesencephalon.

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MATERIALS AND METHODS

Production of Recombinant Human (rh) BDNF and NT-3. A BDNF cDNA clone (1280 base pairs) was isolated from a human fetal brain cDNA library. Large-scale expression of rhBDNF was achieved by transiently introducing a cytomegalovirus-based expression vector containing the human BDNF coding sequence into the human embryonal kidney cell line 293 (13). After 4 days, 5 liters of conditioned medium from the large-scale expression was centrifuged and filtered to remove cells and debris and concentrated 100-fold. The medium was dialyzed into 25 mM sodium borate (pH 9.0) and applied to a DEAE-Sepharose Fast-Flow ion-exchange column (Pharmacia). The column effluent was neutralized (pH 7.0) and this sample was applied to an S-Sepharose ion-exchange column (Pharmacia), washed, and eluted with 25 mM Mops, pH 7.0/0.5 M NaCl.

The presence of rhBDNF in the S-Sepharose salt eluant was measured in 48-hr neuronal survival assays in two types of embryonic (day 10) chicken peripheral ganglionic neurons: spinal sensory neurons of dorsal root ganglia (DRG, lumbosacral region) and nodose ganglion neurons as described (12). The S-Sepharose eluant was dialyzed into 1 M acetic acid/4 M urea, concentrated 10-fold, applied to a Sephacryl S-300 gel-filtration column, and chromatographed in the same buffer. Neuronal survival bioassays of eluant fractions indicated an early broad peak and a later single peak of activity. These two peaks of bioactivity coincided with two peaks of ELISA crossreactivity identified with a polyclonal antibody generated against *Escherichia coli*-produced rhNGF. This antibody was shown to recognize NGF in an ELISA, by radioimmunoprecipitation, and by Western blot analysis (14) and to inhibit the biological activity of NGF on chicken DRG neurons. Characteristics of the antibody and details of the ELISA will be published elsewhere (G. L. Bennett, L.E.B., W. P. Chan, W. L. T. Wong, unpublished observations). An aliquot of the BDNF preparation was submitted to N-terminal sequence analysis by Edman degradation. The sequence of mature rhBDNF, starting with an N-terminal histidine (His^{130}), was obtained for 15 amino acid residues at the 10-pmol level. A mixture of amino acid sequences containing two additional amino acids in equal quantities to that of rhBDNF was obtained for the first five sequencing cycles,

Abbreviations: BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; NGF, nerve growth factor; NT-3, neurotrophin 3; rh, recombinant human; ChAT, choline acetyltransferase; GABA, γ-aminobutyrate; DRG, dorsal root ganglia.

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after which the quantity of amino acids other than those corresponding to the rhBDNF sequence was much smaller than that of rhBDNF. These data indicate that the dominant amino acid sequence is contributed by rhBDNF with minor amino acid or peptidic contamination that in later cycles is reduced to background levels. None of the possible permutations of the sequences of additional amino acids not related to the rhBDNF sequence matched any known protein sequence contained in the National Biomedical Research Foundation data base (June 1990), including human basic fibroblast growth factor (bFGF) or insulin-like growth factors—i.e., growth factors that stimulate cholinergic differentiation in culture (16). Based on the initial recovery (five cycles) of phenylthiohydantoin amino acid derivatives during N-terminal sequencing, the approximate concentration of rhBDNF was 0.7 μg/ml and 1 μg was recovered. A significantly larger recovery of purified rhNT-3 was obtained [20 μg that gave a dominant sequence (12)]. Further details about the human BDNF clone and the expression system will be provided elsewhere (A.R., J.W.W., and K.N., unpublished work).

Cell Cultures. Cultures were prepared from fetal rat (Wistar, day 15–16; Charles River Breeding Laboratories) basal forebrain, containing the cholinergic neurons from septum, diagonal band of Broca, and nucleus basalis and ventral mesencephalon including the dopaminergic nuclei A8, A9 (substantia nigra), and A10 (ventral tegmental area) but not the noradrenergic locus ceruleus. The tissue was gently triturated with an Eppendorf blue pipette tip without using enzymes. The dissociated cells were plated in 16-mm multiwell plates precoated with polyethyleneimine (1 mg/ml, 37°C, overnight). Each well contained 0.5 ml of modified L-15 medium supplemented with 5% heat-inactivated horse serum and 0.5% heat-inactivated fetal bovine serum and the growth factors as specified. In some experiments serum was replaced by the serum-free N2 additions of ref. 15. Modified L-15 was prepared as described (16) by adding various amino acids, vitamins, antibiotics, glucose, and $NaHCO_3$ to Leibovitz's L-15 medium (GIBCO). Plating densities were 4×10^5 cells per cm^2 for basal forebrain cultures and 2×10^5 (low density) or 4×10^5 (high density) cells per cm^2 for mesencephalic cultures. Of these cells 0.5–1% were cholinergic or dopaminergic, respectively. Preparations of rhBDNF or rhNT-3 added to the cultures corresponded to ≤5% of the total volume of medium. After 5–12 days, cultures were taken for determination of choline acetyltransferase (ChAT) activity, for ChAT immunocytochemistry, or for measurement of dopamine or γ-aminobutyrate (GABA) uptake. For initial experiments partially purified rhBDNF from conditioned medium of the kidney cells was used. Equally treated medium from mock-transfected kidney cells served as controls. BDNF and NT-3 were partially purified using a similar protocol to that developed for the purification of rhNGF (17). For BDNF, serum-free medium (0.3 liter), conditioned for 72 hr by line-293 cells transiently transfected with the human BDNF expression vector, was diluted 4-fold with 10 mM Tris·HCl (pH 8.0) and concentrated 10-fold. The sample was then diluted 2-fold with Tris buffer, applied to an S-Sepharose column in the same buffer, washed, and eluted with 0.5 M NaCl. A control sample was generated in identical fashion from conditioned medium isolated from mock-transfected 293 cells. rhBDNF produced by this method was biologically active in embryonic (day 10) chicken DRG and nodose ganglion survival assays (48 hr). rhNT-3 was purified and analyzed as described for rhBDNF. Later, rhBDNF and rhNT-3 purified as described above were used. Biological activity for all tested rhBDNF preparations was determined with DRG and nodose ganglion assays. rhNGF used in the study was provided by the Research Collaborations Group at Genentech and rhbFGF was obtained from Synergen (Boulder, CO). Monoclonal antibody against bFGF was kindly provided by T. Reilly (DuPont). Further methodological details have been published (16–20).

Biochemical Assays. *ChAT assay.* Tissue was homogenized in 250 μl of 50 mM Tris·HCl, pH 6.0/0.3% Triton X-100, and 30 μl of the homogenate was taken for the assay (21). [1-^{14}C]Acetyl-CoA (NEN) concentration was 20 μM and specific activity 4.09 Ci/mol (1 Ci = 37 GBq). Incubation was at 37°C for 20 min. Protein content of the cultures was measured (22) using bovine γ-globulin (Bio-Rad) as a standard.

Dopamine uptake. The method was a modification of that described by Prochiantz *et al.* (23). Cultures were preincubated for 5 min at 37°C with 250 μl of incubation solution [5 mM glucose and 1 mM ascorbic acid in phosphate-buffered saline (PBS)] containing 1 mM pargyline. [^{3}H]Dopamine (37 Ci/mmol) was then added to 50 nM and the cultures were incubated for another 15 min. Blanks were obtained by incubating cells at 0°C. Uptake was stopped by removal of the incubation mixture, followed by five washes with cold PBS. The cultures were lysed in 1% Triton X-100 with 10% perchloric acid and ^{3}H was measured by liquid scintillation counting.

GABA uptake. The uptake was determined as for uptake of dopamine except that preincubation was with aminooxyacetic acid (10 μM) and β-alanine (1 mM) instead of pargyline and the incubation time was only 4 min. [^{3}H]GABA was 100 nM and the specific activity 6 Ci/mmol.

ChAT immunocytochemistry. Cultures were fixed for 30 min in fresh 4% paraformaldehyde in PBS at room temperature and then incubated for 3–5 days at 4°C with a rat monoclonal antibody against ChAT (gift of F. Eckenstein, Oregon Health Science University) diluted 1:4 in 0.1 M sodium phosphate buffer, pH 7.4/5% sucrose/5% bovine serum albumin/0.1% Triton X-100 (PS solution) and normal rabbit serum (1:100 dilution). Subsequent incubations at room temperature were with biotinylated anti-rat antibody (10 μg/ml in PS; Vector Laboratories) and then with an avidin-biotin conjugate of peroxidase (Vectastain, 1:100 in PBS), each for 2 hr. The peroxidase was visualized with diaminobenzidine (1 mg/ml) and H_2O_2 (0.015%) in PBS.

RESULTS

Addition of rhBDNF to basal forebrain cultures increased ChAT activity in a dose-dependent manner (Fig. 1). The rhBDNF-stimulated increase appeared to approach a maxi-

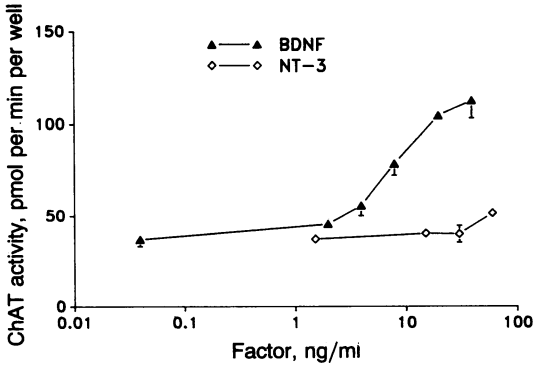

FIG. 1. Dose–response curves for ChAT activity in basal forebrain cultures incubated with rhBDNF (▲) or rhNT-3 (◇). Cultures were grown for 5 days in L-15 medium with 5% horse serum and 0.5% fetal bovine serum. rhBDNF and rhNT-3 were purified from media conditioned by transfected human kidney embryocarcinoma cells. Amounts added to the culture medium were determined by N-terminal sequence analysis. Stock solutions contained rhNT-3 at 3.25 μg/ml or rhBDNF at 0.7 μg/ml. The factors were added to the culture medium 2 days after plating and cultures were taken for measurement of ChAT activity after an additional 3 days. Graph shows means ± SE (n = 4).

mum at the highest concentration of rhBDNF tested (40 ng/ml). In contrast, similar amounts of rhNT-3 in the medium did not enhance ChAT activity (Fig. 1).

The actions of rhBDNF on basal forebrain cholinergic neurons were compared with those of rhNGF and rhbFGF, whose trophic actions on basal forebrain cholinergic neurons *in vitro* have been characterized in detail (16). Basal forebrain cultures were exposed to the three factors after either 2 or 9 days *in vitro* without growth factors. rhbFGF is most effective when given early after plating of the cells, whereas NGF is more effective when applied late *in vitro* (16). In our experiment the response to rhBDNF resembled the rhbFGF response and was clearly different from the rhNGF response (Table 1). rhBDNF produced a greater increase in ChAT activity than rhNGF early during development *in vitro* but, in contrast to NGF, failed to increase this activity when added after 9 days in culture. In another experiment, adding rhBDNF to maximally effective concentrations of rhNGF or rhbFGF increased ChAT activity to levels higher than those reached when rhNGF or rhbFGF was used alone (Fig. 2). However, the effects of the combined factors were not clearly greater than the effect of rhBDNF alone.

In cultures treated with rhBDNF, rhNGF, or rhbFGF, many neurons showed strong ChAT immunoreactivity, whereas control cultures had very few and weakly stained ChAT-immunoreactive neurons (Fig. 3). This finding suggests that rhBDNF stimulates the expression of ChAT in individual cholinergic neurons. The cholinergic cell bodies in growth factor-treated cultures, particularly in the cultures treated with rhNGF or rhBDNF, were clearly larger than in control cultures.

Further experiments aimed at testing the selectivity of the rhBDNF-mediated trophic action on forebrain cholinergic neurons. Selectivity of rhBDNF in septal cultures was compared with that of rhNGF and rhbFGF. Whereas NGF in the forebrain seems to act rather selectively on cholinergic neurons (16, 24–26), bFGF promotes survival of several neuronal populations and also stimulates glial cell proliferation. In basal forebrain cultures, actions of bFGF are reflected in increased cell number, protein content, and GABA uptake (16). Similar to rhbFGF, rhBDNF increased GABA uptake and protein content (Table 2). rhBDNF also slightly increased the cell density in the cultures, as apparent by visual inspection under phase-contrast illumination (Fig. 3). Despite these similarities to the action of bFGF there were clear differences. While the ChAT increase with rhBDNF in repeated experiments appeared always higher with rhbFGF, rhBDNF-mediated increases in protein content and GABA uptake and in cell density appeared always less than those induced by rhbFGF (Table 2, Fig. 3). With rhBDNF treatment fewer cells exhibited a profile typical for non-neuronal cells (i.e., flat cell bodies with no processes or with short processes of variable diameter) than with rhbFGF treatment (Fig. 3*E*). After 3 days of rhbFGF treatment, but not rhBDNF treatment, cells with a morphology typical for non-neuronal cells formed a confluent layer in the culture well (Fig. 3*G*).

Table 1. Immediate vs. delayed administration of rhBDNF, rhNGF, and rhbFGF

Growth factor	ChAT activity, pmol per min per well	
	2 days delayed	9 days delayed
Control	43.3 ± 2.9	164.1 ± 9.5
rhNGF	76.1 ± 7.3*	579.0 ± 67.3*
rhbFGF	90.3 ± 3.2*	176.4 ± 8.2
rhBDNF	97.0 ± 4.7*	139.0 ± 2.2*

Basal forebrain cultures were grown in L-15 medium with 5% horse serum and 0.5% fetal bovine serum. rhNGF (100 ng/ml), rhbFGF (1 μg/ml), or rhBDNF (28 ng/ml) were added 2 days (2 days delayed) or 9 days (9 days delayed) after plating and were present for an additional 3 days. Thereafter (5 and 12 days after plating, respectively), cultures were taken for ChAT assay. rhBDNF, when added to the cultures after 2 days *in vitro* and present in the culture medium for 3 days, increased ChAT activity to 220% of untreated controls; no stimulation was seen when rhBDNF was added after a delay of 9 days. Values are means ± SE (n = 7).
*Different from control group ($P < 0.01$, Student's t test).

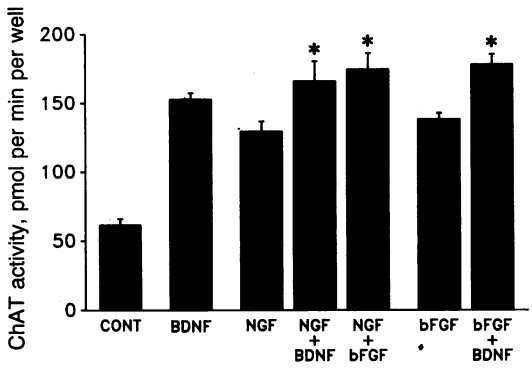

FIG. 2. Comparison of effects of rhBDNF with those of rhNGF and rhbFGF. Basal forebrain cells were grown for 2 days and then exposed for 3 days to maximally effective concentrations of trophic factor(s): rhNGF, 100 ng/ml; rhbFGF, 1 μg/ml; rhBDNF, maximally active concentration of partially purified from transfected human kidney embryocarcinoma cells by S-Sepharose chromatography. Controls (CONT) received an identical preparation from mock-transfected cells. Bars indicate mean ± SE (n = 6–8). Asterisk indicates statistical difference from rhNGF-treated or rhbFGF-treated group ($P < 0.01$, Student's t test).

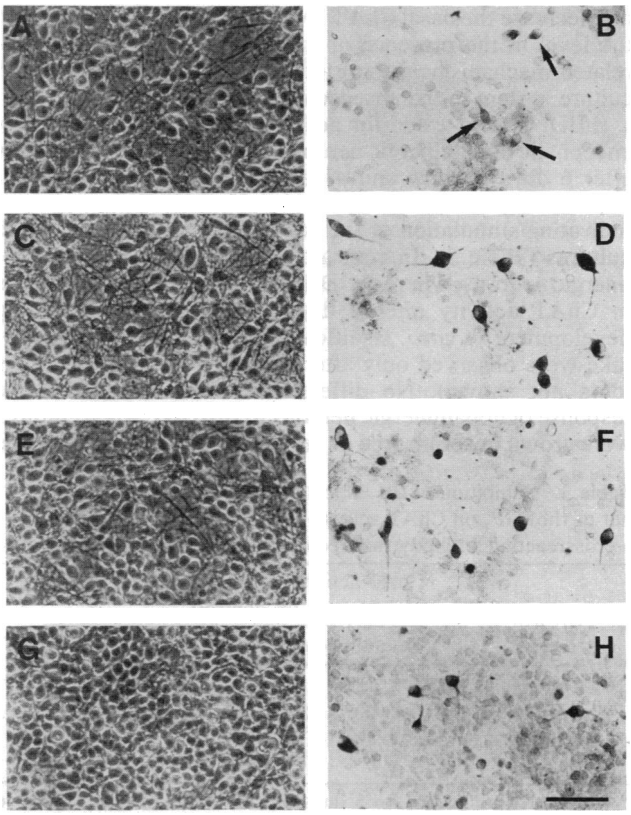

FIG. 3. Phase-contrast microscopy (*Left*) and ChAT immunohistochemistry (*Right*) of rhBDNF-, rhNGF-, and rhbFGF-treated cultures. Basal forebrain cells were grown for 7 days and exposed during the last 5 days to maximally effective concentrations of trophic factors (see legend of Fig. 2). (*A* and *B*) Controls (no addition). (*C* and *D*) rhNGF. (*E* and *F*) rhBDNF. (*G* and *H*) rhbFGF.

Table 2. Effects of rhBDNF, rhbFGF, and rhNGF on GABA uptake, ChAT activity, and protein content in basal forebrain cultures

Growth factor	GABA uptake, fmol per min per well	ChAT activity, pmol per min per well	Protein, μg per well
Control, untreated	1287 ± 78	30.9 ± 2.7	157.5 ± 3.1
rhNGF	1269 ± 40	64.1 ± 0.4*	158.4 ± 0.0
rhbFGF	1660 ± 138*	82.9 ± 1.2*	221.9 ± 1.5*
rhBDNF	1529 ± 113*	93.8 ± 7.9*	211.8 ± 0.3*
rhBDNF mock	Not determined	34.9 ± 1.1	170.6 ± 2.9

Cultures were grown in L-15 medium with 5% horse serum and 0.5% fetal bovine serum. Factors were added 2 days after plating and were present for an additional 3 days: rhNGF, 100 ng/ml; rhbFGF, 1 μg/ml; rhBDNF, maximally active concentration of rhBDNF partially purified by S-Sepharose chromatography (4) from transfected human kidney embryocarcinoma cells; rhBDNF mock, control preparation from mock-transfected cells. Values are means ± SE ($n = 6$).
*Different from control group ($P < 0.01$, Student's t test).

Antibodies clearly differentiated between rhBDNF and rhbFGF stimulation. Specific antibodies against BDNF are not available yet. However, an antibody raised in rabbit against rh-β-NGF produced by a prokaryotic expression system was shown to bind to rhBDNF (see *Materials and Methods*). This antibody completely abolished the effects of rhBDNF and rhNGF on ChAT activity in basal forebrain cultures but did not diminish the response to rhbFGF (Table 3). The rhBDNF response was not impaired by monoclonal antibodies against bFGF, which abolished the rhbFGF-mediated increase in ChAT activity (Table 4). That the antibodies against bFGF did not decrease the basal ChAT level in control cultures or affect the level in the presence of rhBDNF indicates that bFGF-related mechanisms do not affect ChAT activity in our cell culture system in the absence of exogenous bFGF.

rhBDNF was tested for possible trophic actions on mesencephalic dopaminergic neurons. In mesencephalic cultures after 6 days *in vitro* and 4 days treatment with rhBDNF, dopamine uptake was increased by about 100% over control, indicating stimulation of the dopaminergic neurons in these cultures (Table 5). In contrast to the observations on cholinergic neurons, where rhBDNF produced ≈3-fold increases in ChAT activity after 2 days of exposure early during development *in vitro*, significant increases in dopamine uptake were observed only after exposure for at least 4 days (data not shown). No differences were observed in the response of dopaminergic neurons to rhBDNF when cultures were grown in serum-free or serum-containing medium. The magnitude of the stimulation of dopamine uptake was similar to that of GABA uptake and protein content but significantly less than that of ChAT activity in septal neurons.

Table 3. Inhibition of the effects of rhBDNF and rhNGF, but not of rhbFGF, on ChAT activity in basal forebrain cultures by a crossreacting antibody that recognizes NGF and BDNF

Growth factor	ChAT activity, pmol per min per well (% control)	
	No antibody	Anti-NGF/BDNF
Control	33.8 ± 1.5	27.9 ± 1.7
rhBDNF	77.3 ± 2.6* (228)	26.3 ± 2.8* (93)
rhbFGF	60.6 ± 1.0* (179)	54.4 ± 1.0* (174)
rhNGF	50.1 ± 1.4* (163)	28.3 ± 1.4 (100)

Cultures were grown for 3 days in L-15 medium with 5% horse serum and 0.5% fetal bovine serum and treated with growth factors (rhBDNF, 20 ng/ml; rhbFGF, 200 ng/ml; NGF, 2 ng/ml) and antibody for 2 days from the second day of culture. Anti-NGF/BDNF, polyclonal antibodies against *E. coli*-produced rhNGF, were used in cultures at 0.15 mg/ml; for preparation of the antibodies, see *Materials and Methods*. Values are means ± SE ($n = 4$).
*Different from respective control group ($P < 0.01$, Student's t test).

Table 4. Inhibition of the effects of rhbFGF, but not rhBDNF, on ChAT activity in basal forebrain cultures by antibody against bFGF

Growth factor	ChAT activity, pmol per min per well (% control)	
	No antibody	Anti-bFGF
Control	60.8 ± 2.1	63.5 ± 1.6
rhBDNF	137.4 ± 4.4* (226)	143.1 ± 5.0* (225)
rhbFGF	107.2 ± 2.9* (176)	64.8 ± 3.2 (102)

Cultures were grown for 5 days in L-15 medium with 5% horse serum and 0.5% fetal bovine serum and treated with growth factors (concentrations as for Table 3) and antibody for 3 days from the third day of culture. Anti-bFGF, monoclonal antibody against bFGF, was used at 0.1 mg/ml. Values are means ± SE ($n = 6$).
*Different from respective control group ($P < 0.01$, Student's t test).

DISCUSSION

The results suggest that BDNF stimulates development of basal forebrain cholinergic neurons, together with NGF, which a large body of evidence indicates is necessary for normal development of these cells *in vivo* (1–4, 16, 18, 24–30). A physiological role of BDNF in cholinergic function is also suggested by recent reports that, in the adult brain, BDNF mRNA levels are highest in hippocampus and cortex, the target areas of basal forebrain cholinergic neurons (31–33). The availability of rhBDNF and of corresponding molecular biological probes will make it possible to further study its role in the development of the cholinergic neurons. Our finding that BDNF exerts its most pronounced actions on basal forebrain cholinergic neurons during early development *in vitro*, whereas the response to NGF is more pronounced later, suggests that these cells may exhibit different time windows of responsiveness to NGF and BDNF as has been suggested for chick sensory neurons (34). As in peripheral neuronal populations, developmental death may be a feature of cholinergic development and could be regulated by NGF or BDNF. It remains to be established whether BDNF, as shown for NGF under various experimental conditions (18, 35), is able to promote survival and neurite growth of cholinergic neurons.

BDNF and NGF bind to the same protein constituting the well-characterized low-affinity NGF receptors (36), suggesting that actions of the two factors on cholinergic neurons might be mediated by the same receptors. While additivity experiments did not clearly rule out this possibility, it seems unlikely that BDNF in our cultures had its stimulatory effect by activating the high-affinity NGF receptor, since BDNF was more effective than NGF at early culture times whereas NGF was particularly effective later, when BDNF no longer

Table 5. Dopamine uptake in mesencephalic cultures treated with NGF, NT-3, bFGF, or BDNF

Growth factor	Dopamine uptake, fmol per min per well
Control	8.1 ± 2.5
rhNGF	7.8 ± 1.0
rhNT-3	9.3 ± 1.7
rhbFGF	13.8 ± 1.4*
rhBDNF	17.6 ± 2.9*

Cultures were grown for 6 days in L-15 medium with 5% horse serum and 0.5% fetal bovine serum. rhNGF (100 ng/ml), rhNT-3 (60 ng/ml), rhbFGF (1 μg/ml), or rhBDNF (28 ng/ml) was added 2 days after plating and treatment was for 4 days. Values are means ± SE ($n = 6$).
*Different from control group ($P < 0.01$, Student's t test).

increased ChAT activity. Furthermore, binding studies indicate that high-affinity receptors for NGF and BDNF differentiate extremely well between the two factors (36). Approximately 1000-fold higher concentrations of the heterologous ligand were needed to prevent binding of the homologous ligand, whereas in our cultures, effective concentrations of rhNGF and rhBDNF differ <50-fold. However, the binding studies cited above (36) indicate that specific molecular components may be required to confer high-affinity binding and ligand-specific activation of secondary cellular events. If so, our experiments suggest that a BDNF-specific component may be present at early culture times and absent at later times. It remains to be seen whether corresponding temporal changes of responsiveness of the basal forebrain cholinergic neurons to BDNF and NGF also occur *in vivo*.

That rhBDNF increases dopamine uptake in mesencephalic cultures and GABA uptake in basal forebrain cultures indicates effects of BDNF on a spectrum of cells in the brain. This is very different from NGF, whose only clearly established action in the brain is that on cholinergic neurons of the basal forebrain and the striatum. Dopaminergic neuron differentiation is not affected by NGF (16, 37). Effects of NGF on several other neuronal populations have been suggested based on the anatomical distribution of its receptor as determined with immunohistochemical methods (24–26). However, given the existence of novel factors very similar to NGF, these findings may have to be reinterpreted. The protein visualized in these studies may be part of a functional receptor for BDNF or other members of the neurotrophin family (36). Previous studies reported trophic actions of bFGF on dopaminergic neurons in culture, which, however, may require mediation by non-neuronal cells (16, 38). Several investigators provided indirect evidence for the existence of a trophic factor for dopaminergic neurons (39, 40). It remains to be established whether these postulated factors are identical to BDNF. That change induced in dopaminergic and GABAergic parameters is significantly less than that induced in cholinergic parameters suggests that the response of cholinergic neurons to BDNF may be qualitatively different from that of other neurons, including dopaminergic and GABAergic cells. The exact nature of the trophic actions on these populations of cells needs to be established.

The similarity of responses produced by rhBDNF to those of rhbFGF is a surprising result of this study. bFGF supports initial survival and subsequent fiber outgrowth of dissociated rodent fetal neurons in culture. While neurons from many brain regions are affected, the proportion of neurons surviving varies among brain regions, suggesting that subpopulations of neurons are responsive to bFGF (6). Since bFGF lacks a signal sequence typical for released proteins, and since amounts of bFGF in the brain are much larger than those of BDNF and NGF, it has been questioned whether bFGF plays a physiological role as neurotrophic factor and has been proposed that bFGF may act as an "injury factor" released in events involving cellular destruction (4). Our finding that the spectra of neurotrophic responses produced by rhBDNF and by rhbFGF are similar suggests that bFGF released after injury further stimulates biological responses normally produced by BDNF.

Although this study focused on developmental aspects, it is evident from the distribution of mRNA for BDNF in the adult rat brain (31–33) that BDNF, like NGF, may be important for adult brain function. It is intriguing that NGF and BDNF mRNA levels are high in target areas of basal forebrain cholinergic neurons and that the pyramidal layer of hippocampus, which receives a cholinergic input, expresses the two factors (41, 42). It could be speculated that adult cholinergic neurons may exhibit a higher degree of plasticity than other neuronal populations and that NGF and BDNF may be involved in the local regulation of axonal plasticity. Such plastic mechanisms may be required to fulfill a special role in cognitive processes.

We thank the following Genentech colleagues: E. Martin for purification of rhNT-3 and rhBDNF, H. Prasad and F. Wurm for cell transfections and fermentations, A. Shih for partial purification of rhBDNF and neuronal survival assays, and G. Laramee for large-scale plasmid purifications. This study was supported by National Institutes of Health Grant NS22933, National Science Foundation Grant BNS-8996216, and a grant from the National Parkinson Foundation, Miami, FL.

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