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Research report

Differential effects of GDNF and BDNF on cultured ventral mesencephalic neurons

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Abstract

Previous studies have shown that brain derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) can enhance the survival of dopaminergic neurons in the ventral mesencephalon (VM). Here we compared several non-survival functions of the two factors in VM neurons in culture. We found that both BDNF and GDNF elicited an increase in the depolarization-induced release of dopamine, but had no effect on GABA release, in the VM cultures. BDNF, but not GDNF, significantly enhanced the expression of the calcium binding protein calbindin and synaptic protein SNAP25. In contrast, treatment of the cultures with GDNF, but not BDNF, elicited a marked fasciculation of the processes of the VM neurons. Thus, although both act on VM neurons, BDNF and GDNF have distinct functions.

Keywords: GDNF; BDNF; Mesencephalic; Transmitter release; Synaptic protein; Fasciculation

1. Introduction

Two neurotrophic factors, brain-derived neurotrophic factor (BDNF) and Glial cell line-derived neurotrophic factor (GDNF) are known to enhance the survival of dopaminergic neurons in neurons of ventral mesencephalon (VM). We have used cultured VM neurons to investigate whether these neurotrophic factors have effects other than the survival of dopaminergic neurons.

BDNF is a member of the neurotrophin family of secretory proteins, and plays an important role in diverse but specific neuronal responses such as neuronal survival and differentiation [34]. The neurotrophic functions of BDNF are mediated primarily by its high affinity receptor, TrkB tyrosine kinase, although it is also capable of interacting with a low affinity receptor, p75 [5,12]. GDNF belongs to another family of neurotrophic factors which regulates not only the development and function of the nervous system but also the development of the kidney and gastrointestinal system [32,47]. The receptors for GDNF are comprised of two components. There is a signaling component, the c-RET receptor tyrosine kinase, and a high affinity ligand binding component, GDNFR-α [4,10,15,27,28,30,45,54,56,57,61].

Both BDNF and GDNF have been shown to regulate dopaminergic neurons in the ventral mesencephalon. For example, BDNF enhances the survival, morphological differentiation, and dopamine uptake of the VM dopaminergic neurons in culture [24,31,52]. Administration of BDNF in vivo protects VM dopaminergic neurons from lesions induced by 6-hydroxydopamine (6-OHDA) or other neurotoxins [16,33,48,50,51,60], and the BDNF treatment significantly improves locomotion behavior [2,38,65]. Extensive studies have shown that GDNF also exerts a potent regulatory role on the VM dopaminergic neurons. Application of GDNF specifically enhances the survival of dopaminergic neurons in culture [35,36] and in vivo [23,49], increases dopamine synthesis [6], uptake and release [20,22,44].

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GDNF prevents and reverses the degeneration of the VM dopaminergic neurons induced by many chemical and mechanical insults, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [55], 6-OHDA [9,21,29,46], methamphetamine [11], and the lesion of medial forebrain bundle [7]. Moreover, intracerebral injection of GDNF or application GDNF-containing adenoviral construct significantly reduces the death of dopaminergic neurons and improved the locomotion behaviors in rat and monkey models of Parkinson's disease [13,18,63]. Thus, BDNF and GDNF are potentially useful as therapeutic agents to prevent or rescue the degeneration of dopaminergic neurons in Parkinson's disease [17,19,37].

Relatively less is known about the roles of BDNF and GDNF in the non-dopaminergic neurons in the VM. BDNF has been shown to regulate the GABA content and its uptake [1,3,25]. There is no study so far on the role of GDNF on non-dopaminergic neurons in the VM. However, a large proportion of cells in the VM express the BDNF receptor TrkB [64] and/or the GDNF receptors, c-RET and GDNFR-α [58,61,62]. In contrast, less than 1% of VM cells are dopaminergic [13,24,35]. Thus, it is unlikely that the dopaminergic neurons are the only population of cells in the VM that are regulated by BDNF or GDNF.

In this paper, we compared the function of BDNF and GDNF in VM neurons other than the survival of dopaminergic neurons. We found that BDNF and GDNF exhibit differential effects on neurotransmitter release, expression of specific proteins, and neuronal process formation. These studies reveal functional differences of BDNF and GDNF in the ventral mesencephalon.

2. Materials and methods

2.1. Culture preparation

Cultures of ventral mesencephalic neurons were prepared by the published procedure with minor modification [24]. Briefly, the ventral part of the mesencephalon was dissected from embryonic day 14 (E14) of Sprague–Dawley rats, and dissociated in Ca^{2+} and Mg^{2+}-free Hanks balance salt solution (HBSS) containing 0.125% trypsin for 30 min. Cells were then washed with Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were triturated in DMEM/10% FBS, resuspended at the concentration of 0.5 million cells/ml, and plated at one million cells per 35 mm dish (Nunc), or 0.5 million cells per well in 12-well plates. The cells were grown at 37°C, 5% CO_2 and 95% humidity, first in 10% FBS/DMEM, and one day later switched to the serum free medium (SFM), Neurobasal, which contains B27 supplement (Life Technologies). The cultures were grown in SFM and the medium was changed every other day. In some cases for better visualization in immunocytochemistry, cells were plated onto round glass coverslips.

2.2. Immunocytochemistry

VM cultures (16 days old) were washed with 1× phosphate buffer saline (PBS, pH 7.4), fixed with 2% paraformaldehyde for 20 min, and washed twice with PBS. Immunocytochemistry staining was performed as described previously [42]. Briefly, fixed neurons were permeabilized with PBS containing 0.1% Triton X-100 for 1 h. After an incubation in the blocking solution (3% normal goat serum and 3% bovine serum albumin, BSA, in PBS) for 2 h, cells were incubated with primary antibodies at room temperature for 2 h or 4°C for overnight. Primary antibodies used and their dilutions were: anti-tyrosine hydroxylase (TH, Chemicon, 1: 200); anti-GABA antibody (GABA, Chemicon, 1:200); anti-c-RET antibody (c-RET, Santa Crutz, 1:200); anti-TrkB antibody (TrkB, Santa Crutz, 1:200). The specific labeling was visualized using an ABC kit following the manufacturer's instructions. Cells were mounted with Vectorshield mounting solution (Vector). The images of immunohistochemistry were captured by a CCD camera, exported to a Macintosh computer and analyzed by Adobe Photoshop program.

2.3. Dopamine release assay

The VM neurons were cultured in 35-mm dishes in SFM for 8 days and then treated with either 25 ng/ml of BDNF or 1 ng/ml of GDNF or left untreated for an additional 8 days. On the day of the assay, the cells were washed with fresh SFM and then loaded with 1 μCi/ml of [^{3}H] dopamine (specific activity 60 Ci/mmol, NEN) for 1 h at 37°C with 6% CO_2. The cells were then washed four times with SFM and incubated with 1 ml of SFM. For basal dopamine release, 200 μl aliquots were taken after 5 and 10 min of incubation for counting and equal volumes of medium were added back at each time point. For induced dopamine release, 50 mM KCl was added and 200 μl aliquots were removed at 5, 10, 15 and 20 min for counting. Equal volumes of media containing 50 mM KCl were added back at each time point. At the end of the assay, the medium was removed and 1 ml of 0.5 N NaOH was added to each dish and incubated for 1 h at room temperature. The cells were scraped from the dish and 200 μl aliquots were taken for counting. The amount of dopamine release was calculated as the percentage of total counts (counts released/[counts released + counts remained]). For each time point, 3 identical dishes were used to calculate mean ± S.D. Three such assays were performed and similar results were obtained.

2.4. GABA release assay

The VM neurons were cultured in 35-mm dishes in SFM for 8 days and then treated with either 25 ng/ml of BDNF or 1 ng/ml of GDNF or left untreated for an additional 8 days. The GABA release assay was performed essentially as previously described [41]. The cells were washed in Krebs–Ringer Bicarbonate buffer (KRB: KH_2PO_4, 1.2 mM, $NaHCO_3$, 25 mM, NaCl, 137 mM, KCl, 4.8 mM, $CaCl_2$, 2.7 mM, $MgSO_4$, 1.2 mM, glucose, 10 mM, aminooxyacetic acid, 0.1 mM, pH 7.4), and then loaded with 0.5 μM ^{3}H-GABA (2 μCi/dish, NEN) at 37°C for 15 min. The cells were rinsed three times with ice-cold KRB, and then incubated with 1 ml of KRB at 37°C for 15 min. Basal GABA release was measured by counting 200 μl aliquots at every 5 min of incubation and equal volumes of KRB was added back at each time point. For evoked GABA release, 50 mM KCl was added and 200 μl aliquots were removed at 5, 10, 15 and 20 min for counting. Equal volumes of KRB containing 50 mM KCl were added back at each time point. Three identical dishes were used for each time point within an assay, and three assays were performed and mean ± S.D. was calculated.

2.5. Western blot analysis

VM cultures were grown in the SFM for 8 days. BDNF or GDNF was then applied to the cultures for an additional 8 days and the cells were harvested by adding to the cultures 0.1 ml of lysis buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 1 mM EGTA, 50 mM NaF, 1% NP-40, 1% deoxycholate, 0.1% SDS, 1 mM [4-(2-aminoethyl)-benzenesulfluoride hydrochloride], 10 μg/ml leupeptin, 10 μg/ml aprotinin). Cells were harvested with a cell scraper, and incubated on ice for 30 min. Insoluble materials were removed by centrifugation for 10 min at 12,000 × *g*. The protein concentrations of the supernatants

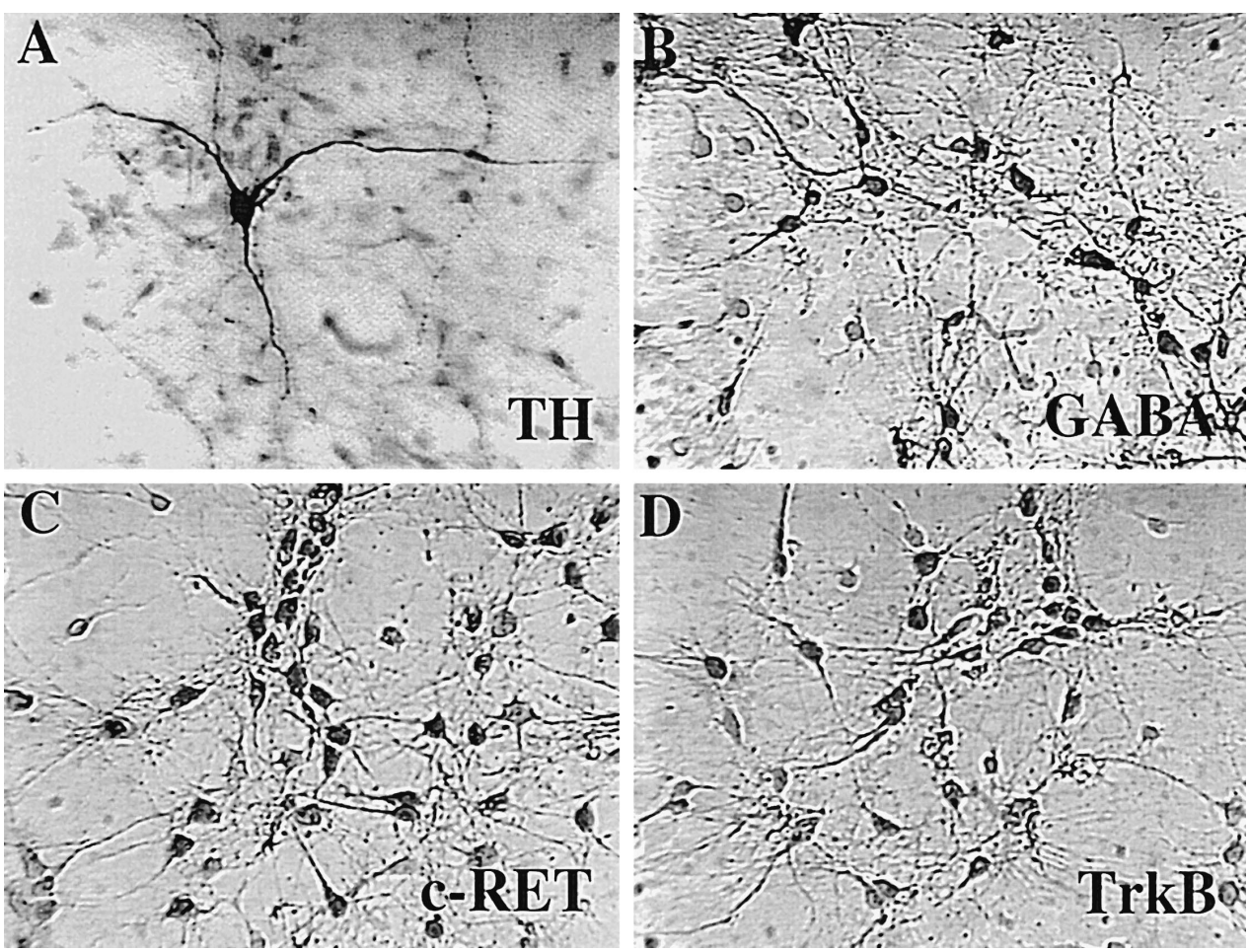

Fig. 1. Characterization of the cultures of ventral mesencephalic neurons. The VM cultures (16 days old) contain dopaminergic and GABAergic neurons, and express both the receptor tyrosine kinases c-RET and TrkB. (A) Dopaminergic neurons immuno-stained with an anti-tyrosine hydroxylase antibody. (B) GABAergic neurons immuno-stained with an anti-GABA antibody (GABA). (C) Expression of c-RET, the receptor for GDNF. Neurons are immuno-stained with an antibody against c-RET tyrosine kinase (c-RET). (D) Expression of TrkB, the receptor for BDNF. Neurons are immuno-stained with an antibody against TrkB tyrosine kinase (TrkB).

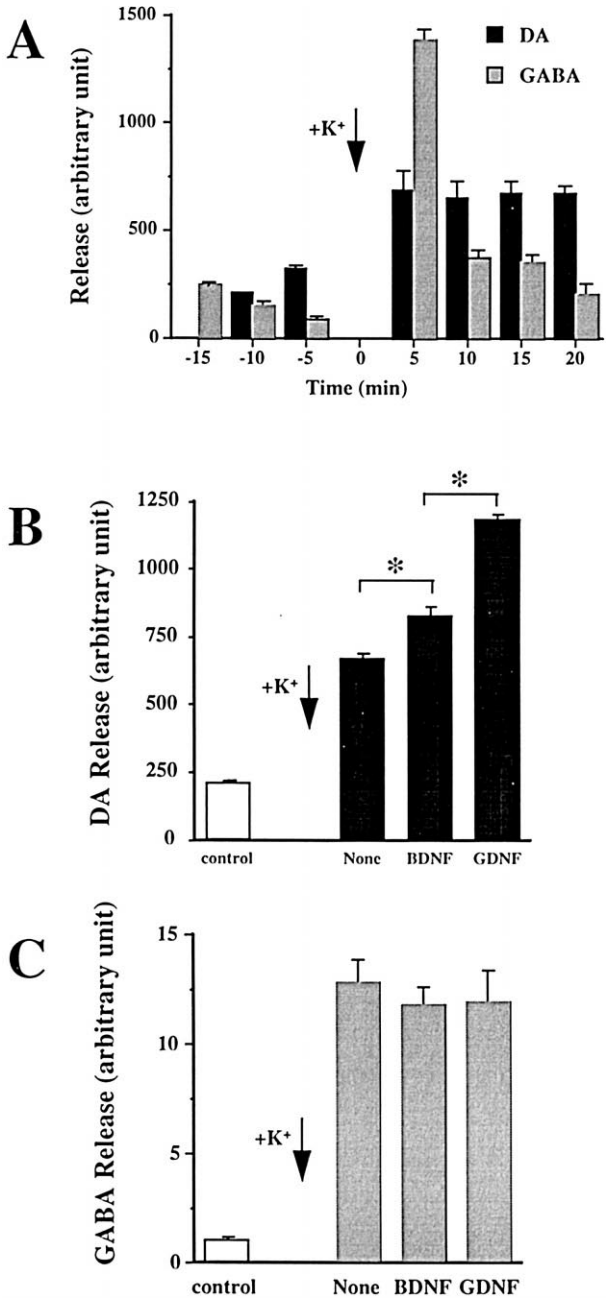

Fig. 2. Effects of BDNF or GDNF on neurotransmitter release in VM neurons. VM cultures were grown in serum-free condition for 8 days and then treated with either GDNF or BDNF for 8 more days. The cells were extensively washed. Then 50 mM KCl was applied to the cultures (arrows) and neurotransmitters released into the media before (basal) and after (induced) KCl application were collected and assayed for dopamine or GABA. Each assay was repeated 3–4 times and a representative assay is shown. All data are presented as mean ± SD. (A) Time course of dopamine (DA) and GABA release. (B) Effects of BDNF and GDNF on dopamine release. Dopamine release within a 20 min time period after KCl application is presented. BDNF-treated group exhibits significantly more KCl-induced dopamine release compared with untreated (None), and GDNF-treated group elicited even further increase of dopamine release (*t*-test, $p < 0.001$). (C) Effects of BDNF and GDNF on dopamine release. There is no difference in KCl-induced GABA release between untreated (None), BDNF-treated and GDNF-treated groups.

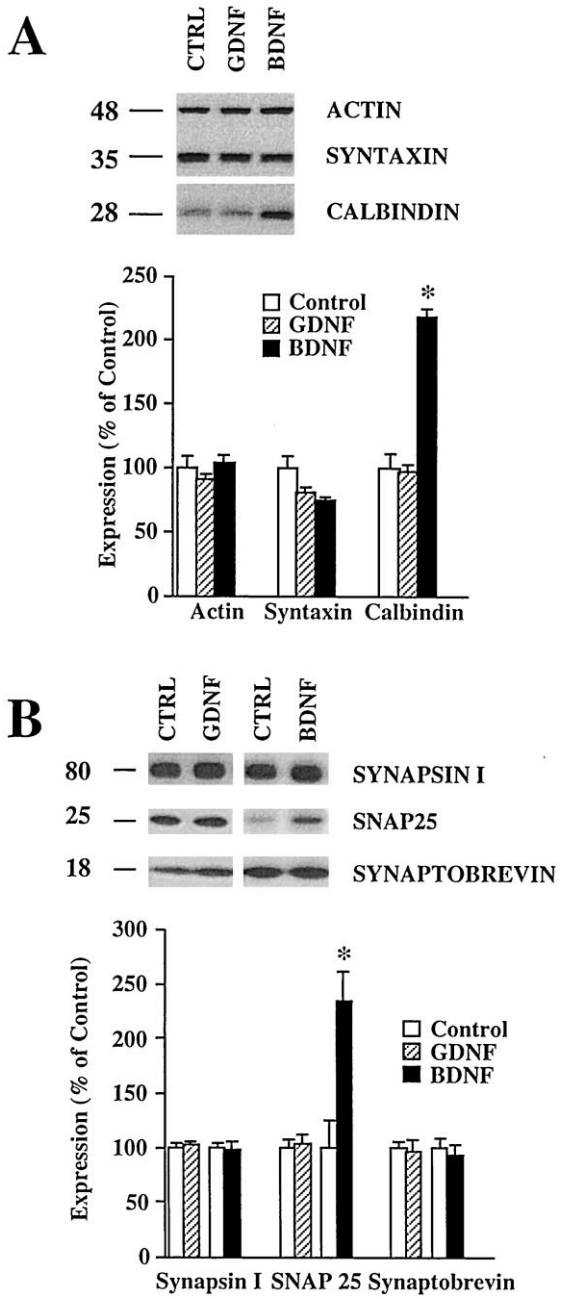

Fig. 3. Differential effects of neurotrophic factors on the expression of neuronal proteins. The VM cultures were grown in SFM for 8 days followed by treatment with either nothing (control), GDNF or BDNF for 8 more days. The cultures were harvested and processed for Western blot to determine the expression levels of Calbindin, SNAP25, actin (A), or syntaxin, synapsin I and synaptobrevin (B). The molecular weights are shown on the left side of the gel. In both A and B, examples of Western blot are shown in the upper panel, and the quantitation of the gels are shown in the lower panel. Each condition was repeated at least 4 times and averaged data are shown.

were determined by the Bio-Rad protein assay based on Bradford method. Equal amounts of proteins were loaded (5–10 μg per lane) onto a 12% SDS-acrylamide gel, and the proteins were separated by electrophoresis, transferred to Immobilon P membrane (Millipore) membrane. The

membranes were first incubated with the blocking buffer (Tris, 0.2 mM, NaCl, 137 mM, 0.2% I-Block and 0.1% Tween-20) for 1 h, and then probed at 4°C overnight with specific antibodies against the following proteins: calbindin, actin, SNAP25 and syntaxin. All primary antibodies were monoclonal IgG or IgM (Chemicon). The immuno-reactive bands were visualized by enhanced chemoluminescence (ECL, Pierce). The ECL signal intensities were quantified using NIH Image based on standard curves of these synaptic proteins.

3. Results

SFM was used to culture neurons derived from E14 ventral mesencephalon. Cultures grown in SFM for 8 days yield virtually pure neurons. Based on morphological criteria (phase bright cells with long processes) and immunocytochemistry (antibody against neuron-specific enolase), more than 95% of cells are neurons. Immunocytochemical staining showed that these cultures contained many GABAergic neurons but only a few tyrosine hydroxylase (TH) positive dopaminergic neurons (Fig. 1A and B). There are many neurons in these cultures that were neither dopaminergic nor GABAergic. We do not know the transmitter phenotypes of these neurons.

We also stained the cultures with antibodies specific for c-RET and TrkB tyrosine kinases, the receptors for GDNF and BDNF, respectively. Many cells were immuno-reactive to c-RET and TrkB antibodies (Fig. 1C and D). Cell counting experiments indicated that $53 \pm 8\%$ of cells were c-RET positive and $57 \pm 12\%$ of cells were TrkB-positive. Moreover, more than half of stained cells expressed both c-RET and TrkB (data not shown), raising the possibility that some neurons are capable of responding to both GDNF and BDNF.

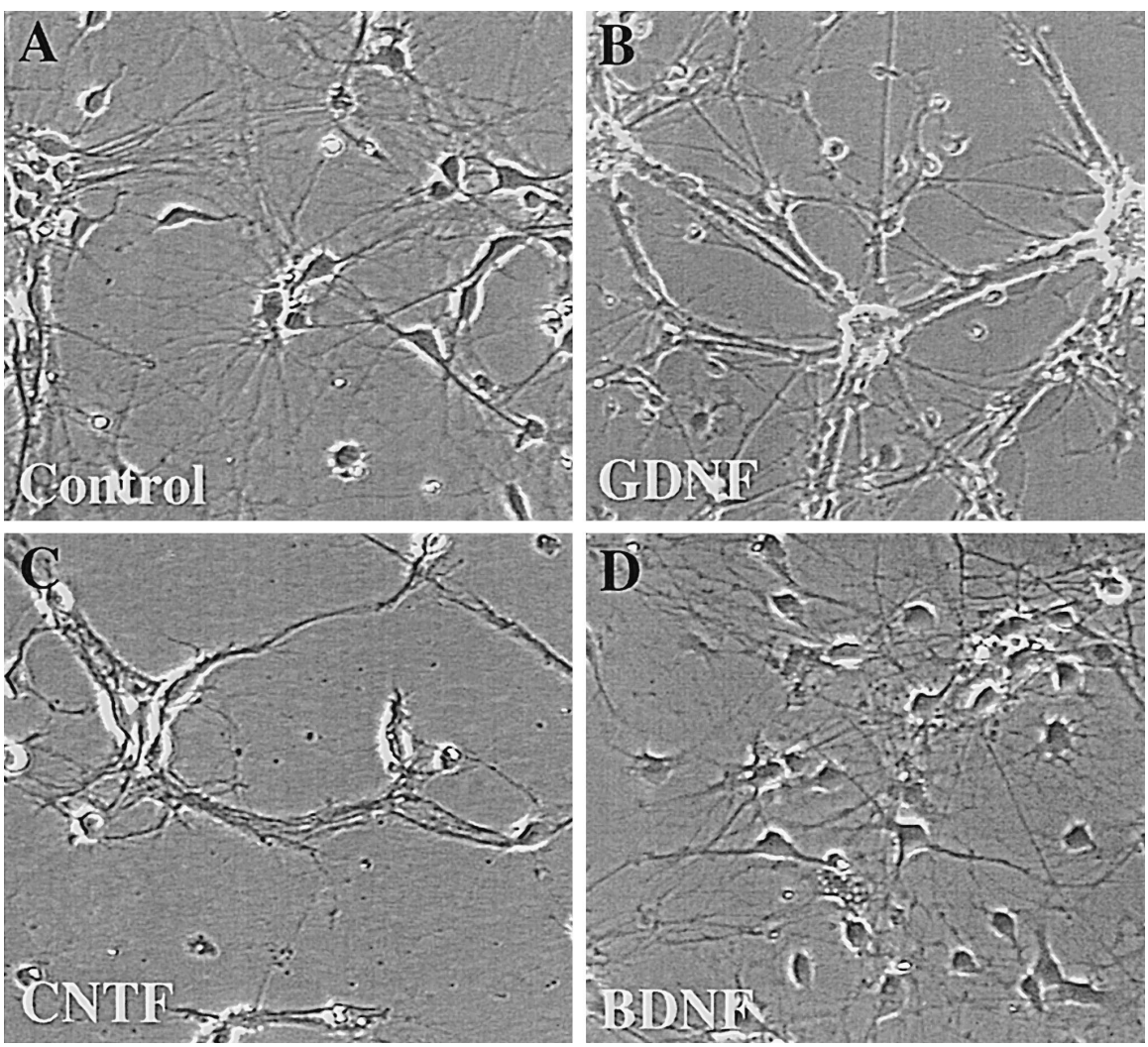

Fig. 4. Fasciculation of neuronal processes in VM cultures. The VM cultures were grown in serum-free conditions and treated with GDNF, CNTF, and BDNF for 10 days. Note the process bundles and aggregation of neuronal cell bodies in CNTF- and GDNF-treated cultures. None of these factors had a significant effect on the total numbers of VM neurons. Fasciculation was evident in cultures treated with GDNF for 4 days, but were more prominent after 8–10 days GDNF treatment. Four such experiments were performed using independent culture preparations, and similar results were obtained.

Three different functional assays were performed to address whether GDNF and BDNF have biological functions other than the survival of VM dopaminergic neurons. We first measured the release of dopamine and GABA, two known neurotransmitters in this system. Cultures of VM neurons (8-day-old) were treated with or without the trophic factors for an additional 8 days. Membrane depolarization induced by application of 50 mM KCl to the culture medium elicited the release of the transmitters (Fig. 2A). Interestingly, the depolarization-induced release of dopamine was sustained up to 20 min after KCl application while that of GABA was transient, returning to lower levels 10 min after application of KCl (Fig. 2A). For dopamine release, basal dopamine release was similar in all groups. Treatment with either BDNF or GDNF elicited an increase in the amount of dopamine released into the culture medium after depolarization. Moreover, high K^+ induced more release of dopamine in GDNF-treated cultures (1 ng/ml) than that in BDNF (25 ng/ml) treated cultures (Fig. 2B). Neither GDNF nor BDNF affected the GABA release (Fig. 2C). Thus, BDNF and GDNF had similar effects on neurotransmitter release in VM neurons.

The second functional assay was to determine whether GDNF or BDNF also regulates the expression of neuronal proteins in VM cultures. Previous studies have shown that BDNF regulates the levels of calbindin and a number of synaptic proteins in the hippocampus [14,26]. We treated 8-day-old cultures with either GDNF (1 ng/ml) or BDNF (25 ng/ml) for an additional 8 days, and measured the levels of several neuronal proteins in these cultures by Western blot. Treatment with BDNF elicited a marked increase in the level of calbindin, without affecting the level of the cytoskeleton protein actin in the VM cultures (Fig. 3A). Moreover, BDNF elicited a specific increase in the level of the synaptic protein SNAP25 (Fig. 3B). Quantitation of the Western blot indicated that BDNF increased the SNAP25 level by 2-fold, while there was virtually no change in other synaptic proteins such as synaptobrevin, synapsin I and syntaxin (Fig. 3). GDNF treatment had no effect on any of the proteins examined. These results indicate that BDNF, but not GDNF, regulates the expression of several specific neuronal proteins.

The third functional assay we used was the morphological changes induced by different trophic factors in VM cultures. Neurons grown in the presence of GDNF exhibited striking and reproducible changes in their morphology (Fig. 4). Neuronal processes were bundled together to form fascicles. Some of the neuronal cell bodies were clustered. The process fasciculation and cell clustering were seldom, if at all, observed in control and BDNF-treated, sister cultures (Fig. 4A and D). Treatment with ciliary neurotrophic factor (CNTF, 20 ng/ml) (Fig. 4C) also elicited process fasciculation, although the effects were less pronounced, compared with GDNF-treated sister cultures (Fig. 4B). We have tried several different plating densities (0.2, 0.5, 0.8 million cells/dish) and found that cell density did not contribute significantly to process fasciculation (data not shown). Thus GDNF, but not BDNF, exerts a specific and reproducible effect on the cellular morphology of VM neurons.

4. Discussion

Extensive studies have shown that both BDNF and GDNF can enhance the survival of the dopaminergic neurons in the ventral mesencephalon [19,24,35,37]. These two factors, however, belong to two completely different families of neurotrophic factors and their receptors and signal transduction pathways are quite distinct [34,40]. The goal of the present study was to compare the functions of these two factors, primarily on non-dopaminergic neurons in VM. We found that BDNF and GDNF exhibit different effects on several biological functions other than the survival of dopaminergic neurons. Our results indicate that BDNF and GDNF elicit distinct biological functions in VM neurons.

We have compared the biological effects of BDNF and GDNF on VM neurons. We found that the two factors share some but not all of their effects. 1) Long-term treatment of the VM cultures with BDNF or GDNF enhanced dopamine release but had no effect on GABA release (Fig. 2). The acute, but not long-term effect of BDNF on dopamine release has been previously reported [8]. Prolonged treatment of midbrain neurons with GDNF has also been shown to increase dopamine release in vivo [20,22] and in culture [44]. We compared the long-term effects of GDNF and BDNF on dopamine release in VM cultures, and found that GDNF had greater effects than BDNF. 2) BDNF, but not GDNF, significantly enhanced the expression of the calcium binding protein calbindin and the synaptic protein SNAP25 (Fig. 3). It has been shown that NT-3 but not BDNF increases the number of calbindin-positive cells in hippocampal cultures [14]. In contrast, GDNF protects against lesion-induced loss of calbindin cells in several brain regions but has no effect on undamaged neurons [39,43]. We found that BDNF elicited a significant increase in calbindin expression (Fig. 3). Within 2 days after BDNF application, the level of calbindin increased (data not shown). We used actin as a measure for total number of neurons per culture, and found that the levels of actin did not change. This result suggests that BDNF increase the amount of calbindin proteins in the VM neurons, rather than increase in the number of calbindin positive cells in VM. Moreover, BDNF also elicited a marked increase in the level of SNAP25, without affecting syntaxin, synapsin I and synaptobrevin. SNAP25 and syntaxin are two proteins associated with the presynaptic membrane, and are known to be involved in synaptic vesicle fusion [53]. These results suggest that BDNF specifically enhance the expression of SNAP25, rather than changing the number of synapses in VM. 3) Treat-

ment of the cultures with GDNF, but not BDNF, elicited a marked fasciculation of the processes of the VM neurons (Fig. 4). Similar fasciculation effects of GDNF has been observed in sympathetic neurons in culture [59]. The mechanism by which GDNF regulates the process fasciculation remains to be established.

The increase in depolarization-induced dopamine release could be mediated by several possible mechanisms. BDNF- or GDNF-treatment could result in a true increase in the ability of dopaminergic neurons to release dopamine at synapses. Alternatively, BDNF or GDNF could enhance the survival dopaminergic neurons, leading to more cells that are capable of releasing DA. It is also possible that some non-dopaminergic neurons could switch to a dopaminergic phenotype upon treatment with the growth factors. A recent study using carbon fiber electrode has demonstrated that indeed, long-term treatment of VM cultures with GDNF results in an increase in the amount of dopamine released per single synaptic bottoms [44]. Our present study is not intended to distinguish these possibilities, but rather to establish the differences between BDNF and GDNF functions on VM neurons.

One of the primary focuses of the present study is on non-dopaminergic VM neurons. Since there are very few TH-positive dopaminergic neurons in the VM cultures, it is unlikely that these neurons contribute substantially to the changes in neuronal morphology and synaptic protein expression. The increase in the expression of calbindin and SNAP25 and appearance of process fasciculation may reflect global effects on VM neurons elicited by BDNF and GDNF, respectively. In fact, more than 50% of the VM cells expressed c-RET and/or TrkB, the receptors for GDNF and BDNF, respectively. This number is much higher than the number of TH-positive dopaminergic neurons in these cultures. Thus, the significance of the present study lies not only on the differential effects of BDNF and GDNF, but also on their non-survival functions of these factors on non-dopaminergic neurons in the ventral mesencephalon.

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