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DIFFERENTIAL SIGNALING OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR AND BRAIN-DERIVED NEUROTROPHIC FACTOR IN CULTURED VENTRAL MESENCEPHALIC NEURONS

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Abstract—In the ventral mesencephalon, two neurotrophic factors, brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor, have been shown previously to have similar effects on the survival of dopaminergic neurons. Here, we compared the signaling mechanisms for brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor, focusing on the mitogen-associated protein kinase and the transcription factor cyclic-AMP responsive element-binding protein. Double-staining experiments indicated that many neurons co-expressed the receptors for glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor, c-RET and TrkB, suggesting that they are responsive to both brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor. Although both brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor induced a rapid phosphorylation of mitogen-associated protein kinase and cyclic-AMP responsive element-binding protein, there were significant differences in the kinetics and pharmacology of the phosphorylation. The phosphorylation of mitogen-associated protein kinase by glial cell line-derived neurotrophic factor was transient; within 2 h, the level of mitogen-associated protein kinase phosphorylation returned to baseline. In contrast, the effect of brain-derived neurotrophic factor was long lasting; the mitogen-associated protein kinase remained phosphorylated for up to 4 h after brain-derived neurotrophic factor treatment. PD098059, a specific inhibitor for mitogen-associated protein kinase kinase, completely blocked the glial cell line-derived neurotrophic factor signaling through mitogen-associated protein kinase, but had no effect on brain-derived neurotrophic factor-induced mitogen-associated protein kinase phosphorylation. Both brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor induced the phosphorylation of cyclic-AMP responsive element-binding protein in the nuclei of ventral mesencephalon neurons. However, PD098059 blocked the cyclic-AMP responsive element-binding protein phosphorylation induced by glial cell line-derived neurotrophic factor, but not that by brain-derived neurotrophic factor.

These results indicate that, although both brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor act on ventral mesencephalon neurons, the two factors have different signaling mechanisms, which may mediate their distinctive biological functions.

Key words: GDNF, BDNF, MAPK, CREB, mesencephalic, PD098059.

It has become widely recognized that different factors may have different effects on the same population of cells through differential activation of signaling mechanisms. Recent studies suggest that the differences in the kinetics of activation of the same signaling molecules contribute to differential functions. For example, in PC12 cells, both epidermal growth factor or nerve growth factor can activate the mitogen-associated protein kinase (MAPK). However, activation of MAPK by the growth factor epidermal growth factor is transient, whereas that by the neurotrophic factor nerve growth factor is sustained.[42] We have examined whether different neurotrophic factors can have differential activation of the same signaling molecules in the same population of primary neurons. Our study focuses on the effects of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) on ventral mesencephalon (VM) neurons in culture.

BDNF and GDNF belong to two different families of neurotrophic factors and regulate diverse neuronal functions.[34,36,51] Both factors have been shown to play a critical role in the development and function of VM dopaminergic neurons. BDNF enhances the survival, morphological differentiation, and dopamine uptake and release of VM dopaminergic neurons in culture.[7,26,32,56] Administration of BDNF *in vivo* protects VM dopaminergic neurons from lesions induced by 6-hydroxydopamine or other neurotoxins, and improves locomotion behavior.[2,17,35,43,52,54,55,61,67] GDNF also exerts a potent regulatory role on VM dopaminergic neurons. Application of GDNF regulates the survival and function of dopaminergic neurons in culture and *in vivo*.[5,23,25,37,38,53] GDNF prevents and reverses the degeneration of VM dopaminergic neurons induced by many chemical and mechanical insults.[6,8,10,24,30,48,58] Moreover, intracerebral injection of GDNF or application of GDNF-containing adenoviral construct significantly reduces the death of dopaminergic neurons and improves the locomotion behaviors in rat and monkey models of Parkinson's disease.[12,19,64] Thus, BDNF and GDNF are potential therapeutical agents to prevent or

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Abbreviations: BDNF, brain-derived neurotrophic factor; CREB, cyclic-AMP responsive element-binding protein; DMEM, Dulbecco's minimum essential medium; EGTA, ethyleneglycolbis(aminoethyl ether) tetra-acetate; FBS, fetal bovine serum; GDNF, glial cell line-derived neurotrophic factor; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; SFM, serum-free medium; VM, ventral mesencephalon.

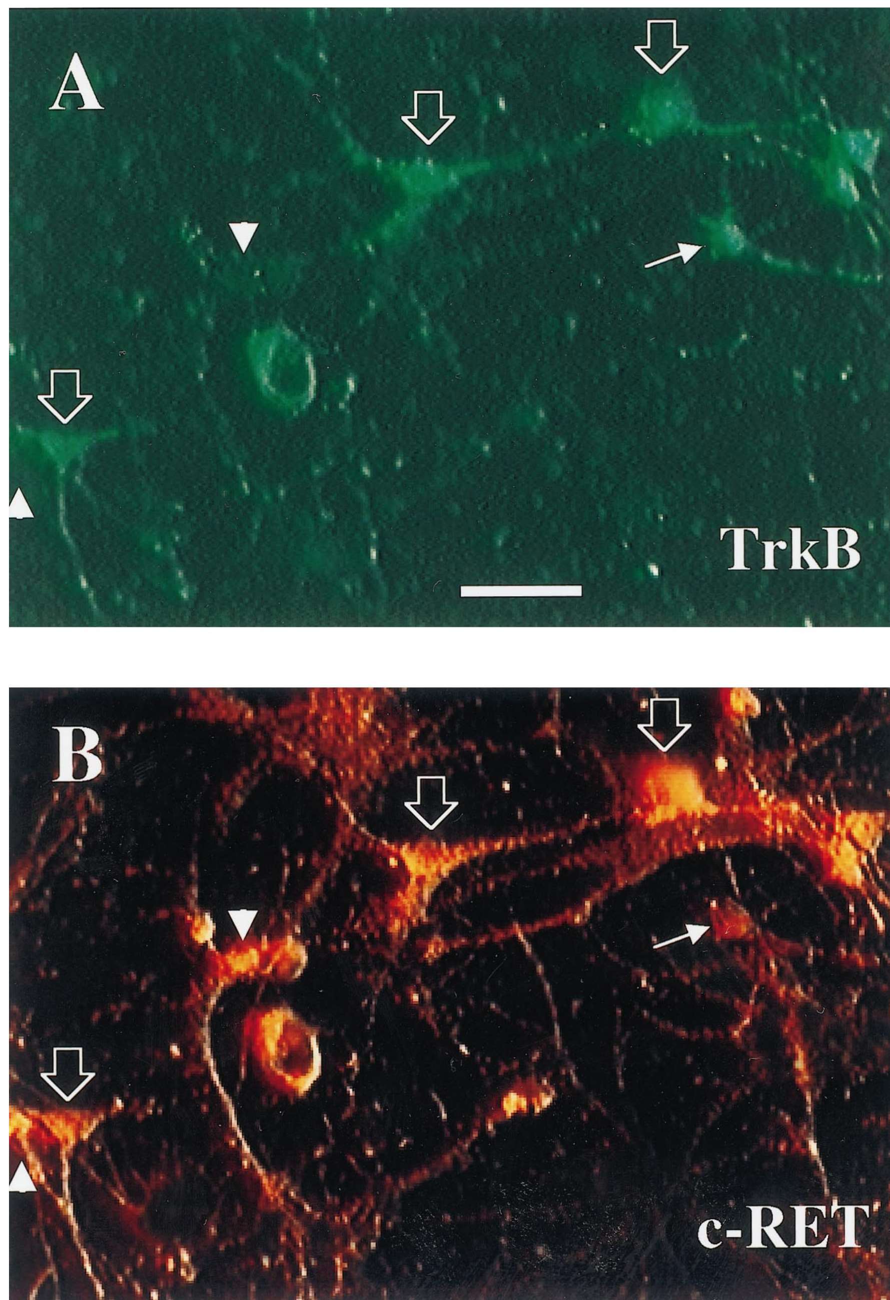

Fig. 1. Co-localization of the BDNF receptor TrkB and the GDNF receptor c-RET on the same VM neurons. VM neuronal cultures were double stained with a polyclonal antibody against TrkB and a monoclonal antibody against c-RET, followed by secondary antibodies conjugated with fluorescein (A, green) for TrkB, or with Cyt3 (B, red) for c-RET, respectively. Long arrows indicate TrkB-positive but c-RET-negative cells, while short arrows indicate TrkB-negative but c-RET-positive cells. Scale bar=20 μm. Note that there are a substantial number of neurons expressing both c-RET and TrkB (open arrows).

rescue the degeneration of dopaminergic neurons in Parkinson's disease.[18,22,39]

The neurotrophic functions of BDNF are mediated primarily by its high-affinity receptor, TrkB tyrosine kinase, although it is capable of interacting with a low-affinity receptor, p75.[4,11] 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.[3,9,15,28,29,31,47,57,59,60,63] The signal transduction mechanisms that BDNF or GDNF use in different populations of neurons are just beginning to be discovered. In several areas of the CNS, binding of BDNF to TrkB has been shown to activate a number of signaling pathways, including the phospholipase C-γ, phophatidylinositol-3$'$ kinase and MAPK pathways (see Refs 21, 40 and 49). GDNF has also been shown to activate MAPK in neuroblastoma cell lines and in sympathetic neurons.[33,62,65] Many neurotrophic functions require transcription and translation of new genes in the target cells. A recent study demonstrated that the immediate-early gene cyclic-AMP response element-binding protein (CREB) is the major mediator for BDNF regulation of gene expression in cortical neurons.[16]

In this paper, the signaling mechanisms for BDNF and GDNF in VM neurons have been characterized.

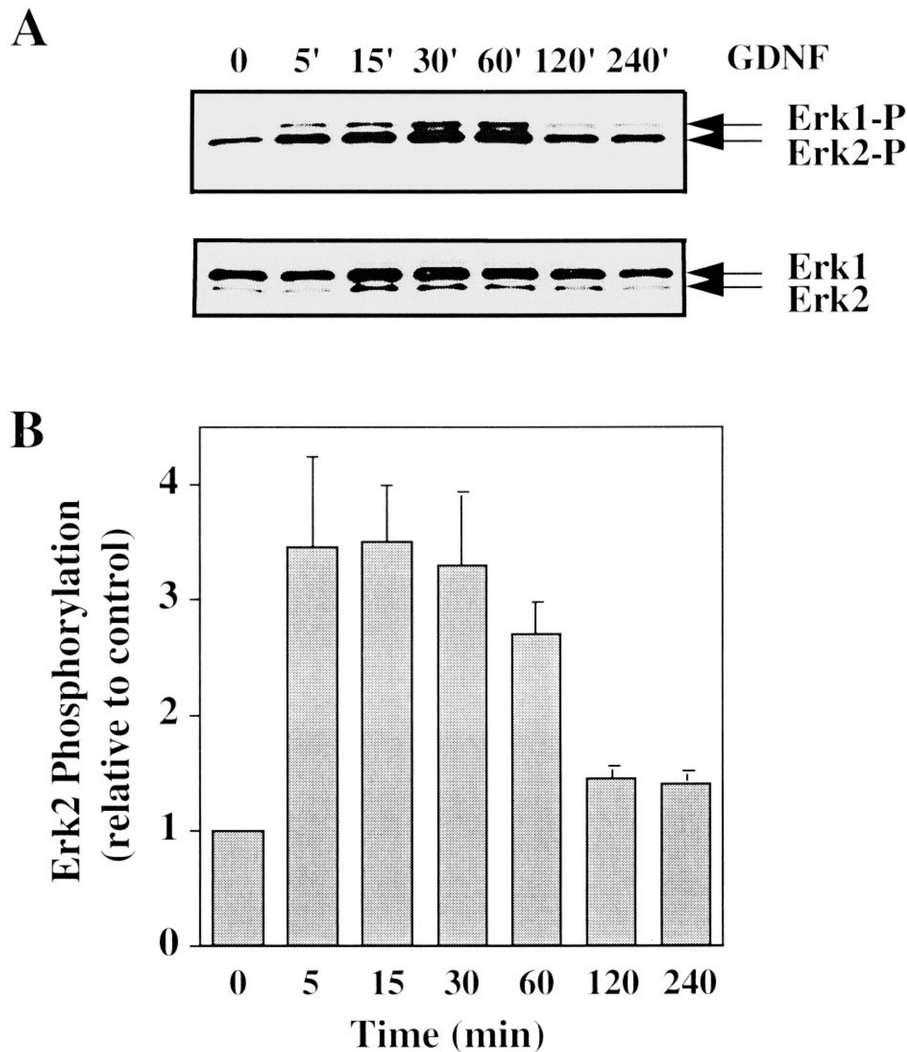

Fig. 2. Time-course of GDNF-induced MAPK phosphorylation in VM cultures. The 10-day-old cultured VM neurons were treated with GDNF (1 ng/ml) and collected at different time-points after GDNF application. The total amount of MAPK (Erk1 and Erk2) was determined by western blot using an anti-Erks antibody, while the activated MAPK was detected by an antibody specific for the phosphorylated Erks (Erk-P). The relative activity of MAPK was quantified by measuring the intensity of the Erk2-P band, normalized to that of the Erk2 band. (A) An example of a western blot showing activated MAPK (upper) and total MAPK (lower). (B) Quantitation of the time-course of MAPK activation. The data are presented as mean ± S.E.M. The values of all the time-points after GDNF application are significantly higher than before GDNF application ("0" time-point). $n=3$. $P<0.05$ (Student *t*-test).

EXPERIMENTAL PROCEDURES

Culture preparation

Cultures of VM neurons were prepared by the published procedure with minor modifications.[26] Briefly, the ventral part of the mesencephalon was dissected from embryonic day 14 (E14) Sprague–Dawley rats (Charles River), and dissociated in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution 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 one 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 12 h later switched to the serum-free medium (SFM), Neurobasal, which contained B27 supplement (Life Technologies). The cultures were grown in SFM for eight to 10 days and the medium was changed every other day. In some cases, for better visualization in immunocytochemistry, cells were plated onto round glass coverslips.

Immunocytochemistry

VM cultures were washed with 1×phosphate-buffered saline (PBS; pH 7.4), fixed with 2% paraformaldehyde for 20 min and washed twice with PBS. Immunocytochemistry staining was performed as described previously.[45] Briefly, fixed neurons were permeablized 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 in PBS) for 2 h, cells were incubated with primary antibodies at room temperature for 2 h or at 4°C overnight. Primary antibodies used and their dilutions were: anti-c-RET antibody (c-RET, Santa Crutz; 1:400); anti-TrkB antibody (TrkB, Santa Crutz; 1:400); anti-phosphorylated CREB antibody (phosphorylated CREB, New England Biolab; 1:500). The specific labeling of phosphorylated CREB was detected using the avidin–biotin–peroxidase complex following the manufacturer's instructions (ABC kit, Vector). For double staining of c-RET and TrkB, cells were incubated with a mouse monoclonal antibody against c-RET (1:400) together with a rabbit polyclonal antibody against TrkB (1:400). The secondary antibodies were Cyt3-conjugated goat anti-mouse immunoglobulin G antibody (1:200) and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G antibody (1:200), respectively. Cells were mounted with Vectorshield mounting solution (Vector). The images of immunocytochemistry or immunofluorescence staining were captured by a CCD camera, exported to a Macintosh computer, and processed and analysed by the Adobe Photoshop program.

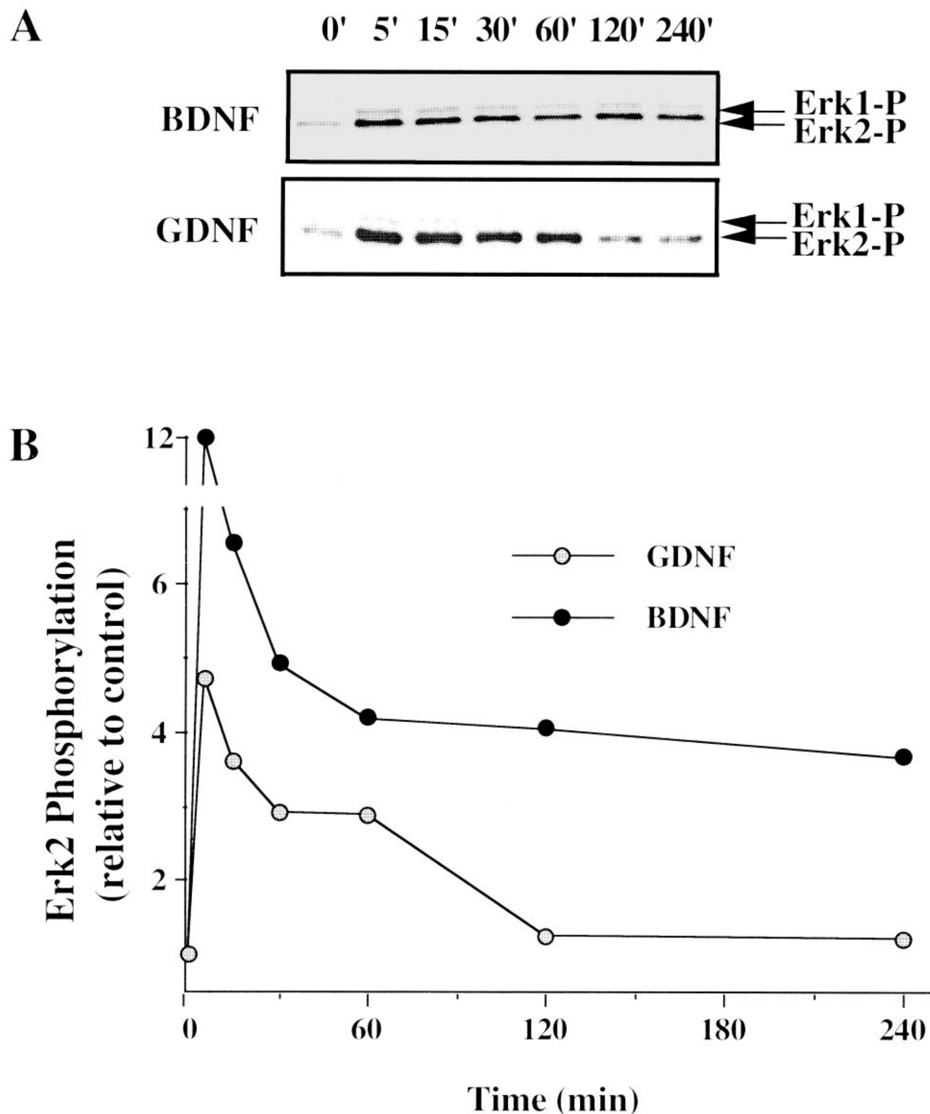

Fig. 3. Kinetics of MAPK phosphorylation elicited by BDNF or GDNF. (A) Examples of the time-courses of MAPK phosphorylation induced by BDNF (upper) or GDNF (lower). (B) Quantitation of the time-courses of MAPK phosphorylation in A. The quantitation of Erk2 phosphorylation levels is adjusted to the amount of Erk2 protein. Note that the phosphorylation of MAPK by GDNF is transient, whereas that by BDNF lasts relatively longer. The time-course of BDNF-induced MAPK activation has been repeated four times, and similar sustained phosphorylation of MAPK was observed.

Western blot analysis of mitogen-associated protein kinase and cyclic-AMP responsive element-binding protein phosphorylation

VM cultures (eight to 10 days) were stimulated with either BDNF or GDNF for the indicated times. The stimulation was terminated 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% sodium dodecyl sulfate, 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 were determined by the Bio-Rad protein assay based on the Bradford method. Equal amounts of proteins were loaded (10 μg per lane) onto a 12% sodium dodecyl sulfate–acrylamide gel, and the proteins were separated by electrophoresis and transferred to Immobilon P membranes (Millipore). The membranes were first incubated with the blocking buffer (0.2 mM Tris, 137 mM NaCl, 0.2% I-Block and 0.1% Tween-20) for 1 h and then probed at 4°C overnight with anti-active MAPK antibody (1:200; Promega), which recognizes phosphorylated MAPK (Erk1-P and Erk2-P). MAPK activation was detected by chemiluminescence detection (ECL, Pierce). The antibody was then stripped and re-probed with an anti-MAPK antibody (1:200; Santa Cruz Biotechnology) to determine the MAPK (Erk1 and Erk2) protein levels. The gels were scanned and the intensities of the bands were quantified using NIH Image software. The relative levels of MAPK phosphorylation in each gel were determined by measuring the intensities of Erk and Erk-P bands, then calculating the ratios of Erk2-P and Erk2 in each lane. Quantitation of MAPK phosphorylation was based on three to four independent experiments (samples); each was repeated at least twice.

For CREB phosphorylation, a similar method was used, except with an antibody against CREB phosphorylated on its serine-133 residue (phosphorylated CREB, 1:500; New England Biolab).

Cell counting

VM cultures (eight to 10 days) were stimulated by the growth factors in the same way as described above. The stimulation was terminated by washing quickly with PBS, followed by fixing with acetone–methanol (1:1) for 3 min. The cultures were stained with the anti-phosphorylated CREB antibody, using the avidin–biotin–peroxidase complex method described above. Phosphorylated CREB was found only in neuronal nuclei. Approximately 2.71% of the total dish area was used to count the number of neurons stained positively

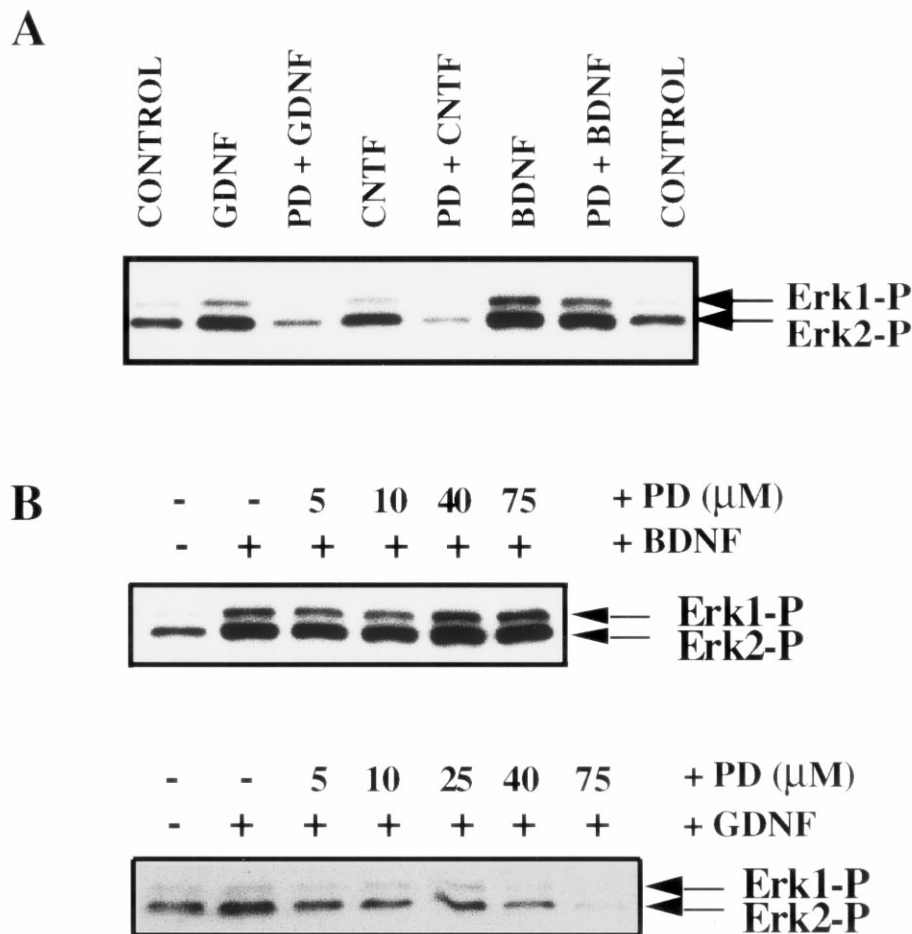

Fig. 4. Effects of PD098059 on MAPK phosphorylation induced by different neurotrophic factors. The VM cultures were pretreated with or without PD098059, an inhibitor of MAPK kinase, for 30 min, followed by application of different neurotrophic factors. The cells were harvested for MAPK measurement 30 min after application of neurotrophic factors. (A) An example of MAPK phosphorylation induced by different neurotrophic factors, and the effects of PD098059 (25 μg/ml). (B) Examples of the effects of PD098059 on MAPK phosphorylation induced by BDNF (upper) and GDNF (lower). VM cultures were treated with different concentrations of PD098059 for 30 min, followed by application of BDNF or GDNF for 30 min. These experiments were repeated four times, and similar results were obtained each time.

for phosphorylated CREB in their nuclei, and three dishes were used for each treatment condition. Similar methods were used to count the number of TrkB- or c-RET-positive cells.

RESULTS

Characterization of mesencephalic neurons in culture

Immunocytochemistry with antibodies specific for TrkB and c-RET tyrosine kinases was used to address the question whether receptors for BDNF and GDNF are expressed in the same VM neurons. Cultures grown in SFM for eight to 12 days yield virtually pure neurons (>95% cells stained positively for neuron-specific enolase). We found that a substantial number of cells expressed c-RET and TrkB (Fig. 1). Cell counting experiments indicated that 53 ± 8% of cells were c-RET positive and 57 ± 12% of cells were TrkB positive. It is also possible that BDNF and GDNF may use different signaling mechanisms in different subsets of neurons. Furthermore, approximately 20% of neurons were double stained by c-RET and TrkB antibodies. These results raised the possibility that at least some neurons are capable of responding to both GDNF and BDNF.

Phosphorylation of mitogen-associated protein kinase by glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor

An antibody that specifically recognizes the phosphorylated form of MAPK was used to monitor MAPK activation. We found that GDNF indeed activated MAPK in VM neurons. As shown in Fig. 2, both Erk1 and Erk2, two isoforms of MAPK, were phosphorylated. Quantitative analysis of the phosphorylation of Erk2 indicated that the enzyme was activated within 5–15 min after cells were exposed to GDNF (1 ng/ml). The MAPK phosphorylation was transient; the levels of Erk2 phosphorylation gradually decreased towards baseline levels within 1–2 h (Fig. 2). Dose–response analysis (ranging from 10 pg/ml to 25 ng/ml) indicated that 1 ng/ml of GDNF was sufficient to induce the maximum amount of MAPK phosphorylation (data not shown).

BDNF also activated MAPK (Fig. 3). Compared with GDNF, however, the effect of MAPK activation lasted longer. MAPK was phosphorylated within 5 min of BDNF application. Quantitative analysis showed that MAPK phosphorylation could last as long as 4 h after

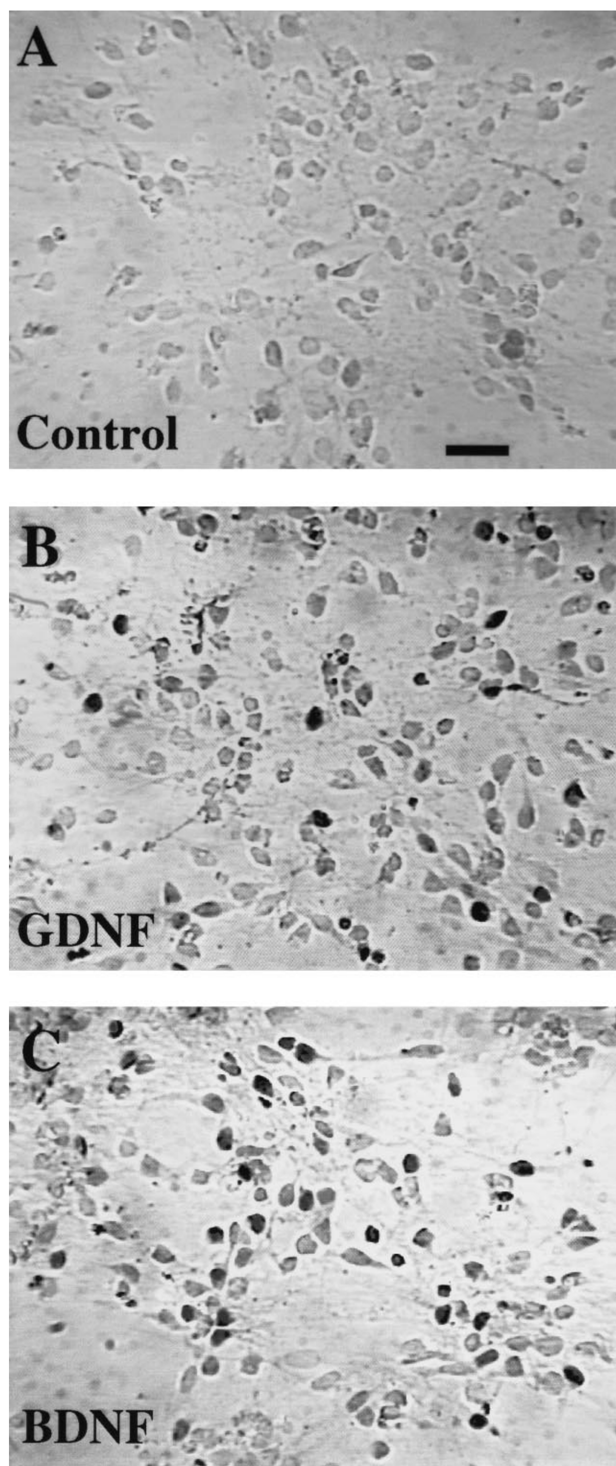

Fig. 5. Effects of BDNF and GDNF on the phosphorylation of the transcription factor CREB. VM cultures were treated with BDNF or GDNF and fixed at different time-points for immunocytochemistry to detect phosphorylated CREB in neuronal nuclei. Examples of phosphorylated CREB staining in control (A) or 15 min after GDNF (B) or BDNF (C) treatment are shown. The dark staining in B and C is phosphorylated CREB-positive nuclei. Scale bar=20 μm.

BDNF (50 ng/ml, equivalent of 2 nM) application (Fig. 3). A lower dose of BDNF (2.5 ng/ml, or 0.1 nM) elicited a similar time-course of MAPK phosphorylation (not shown).

Table 1. Effects of PD098059 on cyclic-AMP responsive element-binding protein phosphorylation elicited by brain-derived neurotrophic factor or glial cell line-derived neurotrophic factor

Control	GDNF	PD098059/GDNF	BDNF	PD098059/BDNF
1.0±1.2	9.6±2.4	2.1±1.6*	17.0±2.9	16.4±2.6

Cultures were treated with PD098059 prior to application of BDNF or GDNF. CREB activation was detected the same way as in Fig. 5. Nuclei stained by anti-phosphorylated CREB antibody were counted. Cells from 2.71% (n=30 fields) of the dish area were counted and a total of three dishes were used for each treatment condition. The data (mean±S.E.M.) are number of phosphorylated CREB-positive cells/field. *The difference between "GDNF" and "PD098059/GDNF" groups is significant (P<0.001, Student t-test).

*Effects of PD*098059 *on mitogen-associated protein kinase phosphorylation by brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor*

To further characterize the difference between GDNF and BDNF in MAPK phosphorylation, we compared their sensitivity to PD098059, a specific inhibitor for MAPK kinase, the enzyme that phosphorylates and activates MAPK.[1,14] The effect of GDNF on MAPK phosphorylation was completely blocked by PD098059 (Fig. 4A). Surprisingly, PD098059 appeared to have no effect on the BDNF-induced MAPK phosphorylation. Dose–response experiments indicated that even 75 μM PD098059 was unable to block the BDNF effect, while 5 μM PD098059 was sufficient to block the GDNF effect (Fig. 4B, C). These results indicate that BDNF and GDNF may use different pathways to activate MAPK. Interestingly, ciliary neurotrophic factor, a neurotrophic factor of a different family,[27] also activated MAPK in these cultures. MAPK phosphorylation induced by ciliary neurotrophic factor was completely blocked by PD098059 as well (Fig. 4A).

Differential effects of brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor on the phosphorylation of cyclic-AMP responsive element-binding protein

Growth factor-stimulated CREB activation can be mediated by the MAPK pathway.[16,50,66] MAPK activates RSK2 (or CREB kinase), which in turn phosphorylates CREB at serine-133.[66] The different effects of BDNF and GDNF on MAPK may lead to a difference in CREB activation. To test this idea, we performed immunocytochemistry on cultures treated with BDNF or GDNF, using an antibody that specifically recognizes CREB phosphorylated on its serine-133 residue. We found that application of BDNF or GDNF elicited a rapid increase in the staining of phosphorylated CREB in the nuclei of VM neurons (Fig. 5). In control conditions, there are very few phosphorylated CREB-positive cells and staining was very weak (Fig. 5A). Within 15 min after application of GDNF or BDNF, dark staining appeared in the nuclei of these neurons (Fig. 5B, C). Cell counting experiments indicated that the time-courses of CREB phosphorylation were similar in GDNF- and BDNF-treated cultures (data not shown). However, there were more phosphorylated CREB-positive nuclei in cultures treated with BDNF, compared to those treated with GDNF (Table 1). Pretreatment of the cultures with PD098059 (5 μM) prevented the phosphorylation of CREB induced by GDNF, but not that by BDNF (Table 1). Western blot using the same

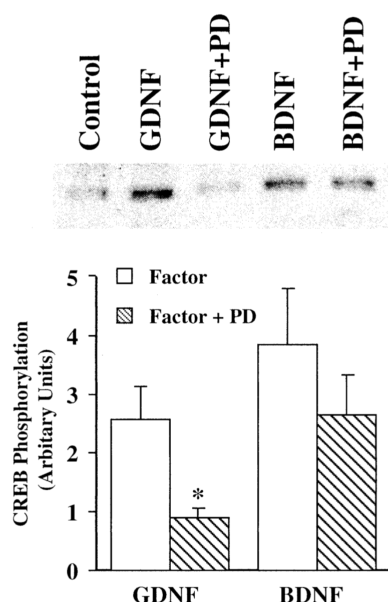

Fig. 6. Quantitation of the effect of PD098059 on CREB phosphorylation. Western blots were performed to detect phosphorylated CREB (pCREB). (A) Examples of western blots. (B) Quantitation of four independent experiments. *Significantly different ($P<0.05$, Student *t*-test). Note that the phosphorylation of CREB by BDNF is slightly reduced by PD098059, whereas that by GDNF was completely blocked by PD098059.

antibodies revealed similar results: PD098059 cannot reverse the CREB phosphorylation induced by BDNF (Fig. 6). Thus, BDNF and GDNF may use different signaling mechanisms to activate the transcription factor CREB.

DISCUSSION

Extensive studies have shown that both BDNF and GDNF can enhance the survival of dopaminergic neurons in the VM.[22,26,37,39] These two factors, however, belong to two completely different families of neurotrophic factors.[36,44] The goal of the present study was to compare the signaling mechanisms of the two neurotrophic factors in VM neurons. We found that, although BDNF and GDNF both activate MAPK and CREB, there are a number of important differences in the way that MAPK and CREB are activated. These results provide new insights on the role of BDNF and GDNF in VM neurons, and suggest that BDNF and GDNF may elicit distinct biological functions via different modes of the same signal molecules.

Depending on the cell types examined, trophic factors are capable of activating a variety of signaling pathways. The specific signaling pathway mediating a particular function of a factor in a certain neuronal population needs to be investigated individually. BDNF has been shown to activate the MAPK and phospholipase C-γ pathways in hippocampal neurons in culture.[40,41] A recent report showed that CREB is a major mediator of BDNF responses in hippocampal slices.[16] We report here that BDNF-induced phosphorylation of both MAPK and CREB in cultured VM neurons, a group of cells quite different from hippocampal neurons in their origin, property and cellular functions. Using a specific antibody that recognizes the phosphorylated forms of MAPK, we found that BDNF rapidly induced the phosphorylation of MAPK in VM neurons. The signaling mechanisms for GDNF in CNS neurons have not been investigated, although studies using cell lines and sympathetic neurons have shown that GDNF is capable of activating MAPK.[33,62,63,65] Our results indicated that GDNF can activate both MPAK and CREB in VM neurons.

Most interestingly, we found that BDNF and GDNF activate MAPK and CREB in distinct manners. (1) GDNF induced a transient, while BDNF induced a sustained, phosphorylation of MAPK (Fig. 3). The difference in time-course of MAPK phosphorylation by BDNF and GDNF was not due to differences in concentrations of the factors used in the experiments. Transient phosphorylation of MAPK was observed when a saturated concentration of GDNF (1 ng/ml) was used. Sustained phosphorylation of MAPK was induced by BDNF when either high (50 ng/ml) or low (2.5 ng/ml) concentrations were used. (2) GDNF-induced MAPK phosphorylation can be blocked by PD098059, a specific inhibitor for MAPK kinase, while that induced by BDNF cannot (Fig. 4). (3) CREB phosphorylation elicited by GDNF can also be reversed by PD098059, while that by BDNF cannot (Table 1). Differential activation of MAPK and CREB by BDNF and GDNF suggests that the two factors may have different functions in VM neurons.

It is interesting that BDNF-induced phosphorylation of MAPK and CREB in the VM neurons is resistant to PD098059 block. MAPK is the only known substrate of MAPK kinase, a dual kinase that phosphorylates MAPK on a threonine as well as a tyrosine residue.[13,46] PD098059 specifically inhibits the activity of MAPK kinase, and therefore prevents the activation of MAPK.[1,14] A recent study, however, demonstrated the prolonged activation of MAPK through MAPK kinase-independent pathways.[20] Thus, it is possible that BDNF activates MAPK by other mechanisms when the Ras/MAPK kinase pathway is blocked by PD098059. Alternatively, BDNF could elicit an inhibition of a phosphatase that dephosphorylates MAPK, leading to PD098059-resistant, prolonged phosphorylation of MAPK. While specific mechanisms remain to be determined, it is possible that sustained activation of MAPK could contribute to the BDNF-induced, PD098059-resistant activation of CREB in nuclei.

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