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Comparison of Survival-Promoting Effects of Brain-Derived Neurotrophic Factor and Neurotrophin-3 on PC12h Cells Stably Expressing TrkB Receptor[1]

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We obtained two PC12h cell lines, PC-pAB1 and PC-pAB2, stably expressing TrkB receptor and investigated the effects of BDNF and NT-3 in these cell lines. The cells differentiated into neuron-like cells in response to BDNF as well as NGF, neurite extension being more rapid in the former case. These TrkB-expressing cells also extended neurites in response to NT-3, which is a nonpreferred ligand of TrkB. Next, we examined the survival-promoting effects of NGF, BDNF, and NT-3 under apoptotic conditions of oxygen toxicity in naive cells and NGF deprivation in differentiated cells. In both cases, BDNF prevented cell death similarly to NGF. NT-3 prevented cell death induced by oxygen toxicity in naive cells, but not that induced by NGF deprivation in differentiated cells. NT-3 induced the tyrosine phosphorylation of TrkB in naive cells, but not in differentiated cells. These results indicate that NT-3 has survival-promoting effects on naive TrkB-expressing PC12h cells, but not on differentiated cells because of its inability to induce the tyrosine phosphorylation of TrkB.

Key words: neurotrophin, PC12 cells, signal transduction, survival promotion, TrkB receptor.

Nerve growth factor (NGF) is the prototype of a family of NGF-related proteins known as the neurotrophins, which include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and neurotrophin-6 (NT-6) (*1*). The neurotrophins bind to and activate specific receptor tyrosine kinases of the Trk family (*2, 3*). TrkA, TrkB, and TrkC are receptors for NGF, BDNF, and NT-3, respectively. BDNF and the second ligand NT-4/5 bind to the TrkB receptor as preferred ligands. NT-3 also binds to the TrkB receptor as a nonpreferred ligand (*4*).

The rat pheochromocytoma cell line PC12 has proved to be a useful model for investigating the mechanisms of action of the neurotrophins (*5*). PC12 cells differentiate into sympathetic neuron-like cells in response to NGF. PC12 cells treated with NGF show marked cellular changes including extension of neurites, cessation of mitosis, increased neurotransmitter biosynthesis and development of Na^+ and Ca^{2+} channels.

NGF and BDNF bind to and activate TrkA and TrkB, respectively. The activated receptor tyrosine kinases are autophosphorylated on tyrosine residues and initiate intracellular signaling which culminates in specific programs of gene expression and cellular responses (*3*). The tyrosine-phosphorylated Trk receptor serves as a scaffolding for the recruitment of several enzymes and effectors, including phospholipase C-γ (PLC-γ) (*6-8*), phosphatidylinositol 3-kinase (PI3-K) (*9, 10*), and the adapter protein Shc (*8, 10*). Each of these molecules activates distinct pathways which have different functions. The Ras-MAP kinase pathway initiated by Shc is considered to be involved in neuronal differentiation induced by neurotrophins, while the PI3-K pathway may be involved in neuronal survival promoted by these molecules (*11*). The Ras-MAP kinase pathway is composed of Shc, Grb2, Sos, Ras, Raf, MAP kinase kinase (MAPKK, also known as MEK), and MAP kinase (MAPK, also known as Erk) (*3*). Shc is phosphorylated on tyrosine residues by the activated Trk receptors and MAPK is

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Abbreviations: AChE, acetylcholinesterase; BDNF, brain-derived neurotrophic factor; DMEM, Dulbecco's modified Eagle's medium; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MEK, MAPK-ERK kinase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide; NGF, nerve growth factor; NT-3, neurotrophin-3; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC-γ, phospholipase C-γ; $p70^{S6K}$, p70 ribosomal protein kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Shc, Src homologous and collagen protein; SNT, Suc1-associated neurotrophic factor-induced tyrosine-phosphorylated target; Sos, Son of Sevenless; STAT, signal transducers and activators of transcription.

phosphorylated by the activated MAPKK.

Neurotrophins have survival-promoting effects on neuronal cells. NGF is known to protect PC12 cells from apoptosis induced by various stresses, including NGF and/or serum deprivation (*12*) and oxygen toxicity (*13*). BDNF protects cerebellar granule neurons from low K^+-induced apoptosis (*14*). As mentioned above, PI3-K may be important for neuronal survival promoted by neurotrophins, although the exact mechanism of its survival-promoting effect remains to be determined (*15*). In this report, we compared the neurotrophic effects of BDNF and NT-3 on two PC12-derived cell lines stably expressing TrkB receptor. NT-3 had a survival-promoting effect on naive TrkB-expressing cells, but this effect disappeared during the neuronal differentiation of TrkB-expressing PC12h cells.

MATERIALS AND METHODS

Materials—Recombinant human BDNF and NT-3 were kindly provided by Amgen/Regeneron Partners. NGF was prepared from male mouse submandibular glands according to the method of Bocchini and Angeletti (*16*) modified according to Suda *et al.* (*17*). The pAGE123 vector was a generous gift from Dr. N. Sato (Osaka University Medical School). To construct PC12h cells stably expressing TrkB, full-length rat *trk*B cDNA subcloned in the pAGE123 vector was transfected into PC12h cells. The pRc/RSV vector was purchased from Invitrogen. Plasmids were purified by QIAGEN-tip (QIAGEN). Anti-pan-Trk antiserum was raised in rabbits against a synthetic peptide corresponding to the 14 carboxyl-terminal amino acids of human TrkA (*18, 19*). The anti-phosphotyrosine monoclonal antibody 4G10 was purchased from Upstate Biotechnology. Anti-phosphorylated MAP kinase antibody was purchased from New England Biolab. Anti-TrkB monoclonal antibody was kindly provided by Dr. S. Koizumi (Novartis Pharma, Takarazuka).

Cell Culture—The PC12h cell line is a subclone of PC12 cells which, like the parental cells, differentiates into neuron-like cells in response to NGF (*20*). PC12h cells and PC12h cells stably expressing TrkB were maintained in 75 cm^2 flasks (Costar) using Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 5% (v/v) precolostrum newborn calf serum (PNCS, Mitsubishi Kagaku) and 5% (v/v) heat-inactivated (56°C, 30 min) horse serum (HS, Gibco). The cells were cultured in 100-mm collagen-coated Falcon dishes (surface area, 55 cm^2; to examine the tyrosine phosphorylation of Trks), 60-mm collagen-coated Falcon dishes (surface area, 21 cm^2; to detect the tyrosine phosphorylation of MAP kinase), collagen-coated 6-well Costar plates (surface area, 9 cm^2; to assay AChE activity), collagen-coated 24-well Costar plates (surface area, 2 cm^2; to observe neurite outgrowth), or collagen-coated 48-well Costar plates (surface area, 1 cm^2; to detect survival effects). Cells were cultured in DMEM supplemented with 5 μg/ml human transferrin, 5 μg/ml bovine insulin, and 20 nM progesterone (TIP/DMEM) (*21*) (to observe neurite outgrowth and to assay AChE activity), or in DMEM containing serum (to detect the tyrosine phosphorylation of Trks and MAP kinase). To detect the tyrosine phosphorylation of Trks and MAP kinase, the medium was changed to that without serum 2 h before adding NGF, BDNF or NT-3.

Construction of PC12h Cells Stably Expressing TrkB—Recombinant pAGE123 containing the full-length rat *trk*B cDNA was used as the TrkB-expression plasmid, and transfection was performed by LipofectAMINE (Gibco BRL). PC12h cells were plated onto polyethyleneimine (PEI)-coated 6-well plates at a density of 0.75×10^4 cells/cm^2 and cultured in DMEM containing 5% (v/v) PNCS and 5% (v/v) HS for 1 day. The medium was changed to 1 ml of TIP/DMEM containing 5 μg of TrkB-expressing plasmid and 90 μl LipofectAMINE per well. The cells were cultured for 5 h in a 10% CO_2 incubator, then an equal volume of DMEM containing 10% (v/v) PNCS and 10% (v/v) HS was added to the culture. After incubation for 1 day, the cells were detached from the plates by pipetting. The cells were replated at a lower density and cultured in DMEM containing 5% (v/v) PNCS, 5% (v/v) HS, and 500 ng/ml G418 (Gibco BRL) for about 2 weeks. Resistant colonies were assayed for differentiation in the presence of 50 ng/ml BDNF, and two stable clones, designated PC-pAB1 and PC-pAB2, were seleted after two limiting dilutions.

Assay of Acetylcholinesterase (AChE) Activity—The cells were washed once with phosphate-buffered saline and lysed by sonication in ice-cold 20 mM Tris-HCl, pH 7.5, containing 0.5% Triton X-100, 10 mM $MgCl_2$, and 150 mM NaCl. The lysates were assayed for acetylcholinesterase (AChE) activity using 0.5 mM acetylthiocholine (Sigma) and 0.4 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (*22*). The enzyme reaction proceeded at 37°C and was stopped by addition of an equal volume of 5% SDS. The absorbance was measured photometrically at 412 nm. The specific activity was calculated on the basis of protein concentrations in the enzymatically assayed samples determined with a BCA protein assay kit (Pierce).

Detection of Survival-Promoting Effects of Neurotrophins—To detect survival effects of neurotrophins on apoptosis induced by oxygen toxicity in naive cells, cells were plated on 48-well plates at a density of $2\text{-}4 \times 10^4$ cells/cm^2 and cultured in DMEM containing 5% (v/v) PNCS and 5% (v/v) HS for 1 day. The medium was changed to TIP/DF medium (DME : Ham's F12 = 1 : 1) in the absence or presence of 50 ng/ml NGF, BDNF, or NT-3, and cells were incubated in a 50% O_2 (5% CO_2) incubator for 2.5 days. The number of viable cells was assessed by MTT assay.

To detect survival effects on apoptosis induced by NGF deprivation in differentiated cells, cells were plated on collagen-coated 24-well plates at a density of $0.5\text{-}1 \times 10^4$ cells/cm^2 and cultured in DMEM containing 5% (v/v) PNCS and 5% (v/v) HS for 1 day. The medium was changed to TIP/DF medium (DME : F12 = 1 : 1) in the presence of 50 ng/ml NGF, and cells were incubated in a 5% CO_2 incubator for 7 days. Cultures were refed every other day. The cells were washed once with DF medium and cultured in DF medium in the absence or presence of 50 ng/ml NGF, BDNF, or NT-3 for 4 days. Cultures were refed every other day. The number of viable cells was assessed by MTT assay.

MTT Assay—The MTT assay was performed according to a modification (*14, 23*) of the original procedure (*24*). Briefly, the tetrazolium salt MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide] was added to the cultures at a final concentration of 1 mg/ml. After incubation at 37°C for 2 h, the assay was stopped by adding lysis buffer consisting of 20% (w/v) SDS and 50% (v/v)

N,N-dimethyl formamide, pH 4.7. The absorbance was measured photometrically at 570 nm after incubation at 37°C overnight.

Immunoprecipitation and Immunoblotting—PC12h cells stably expressing TrkB were washed once with ice-cold Tris-buffered saline (TBS), then lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 20 mM Tris-HCl, pH 7.5, 1 mM PMSF, and 5 μg/ml aprotinin to detect the tyrosine phosphorylation of Trks, or in a buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 20 mM Tris-HCl, pH 7.5, 1 mM PMSF, and 5 μg/ml aprotinin to quantify TrkB receptor expression. Lysates were centrifuged at 10,000× *g* at 4°C for 30 min, then the protein concentration of the clarified lysate was determined by the BCA protein assay. Antibody wad added to the lysates in excess: anti-pan-Trk antiserum (10 μl) for detection of Trk tyrosine phosphorylation or quantitation of the TrkB receptor was added and incubated at 4°C for 1-3 h. Protein A-Sepharose (30 μl) was then added, and the tube was rotated at 4°C for 30-60 min. The immune complexes were pelleted by centrifugation at 10,000× *g* at 4°C for 1 min, then washed 3-5 times with lysis buffer. The immune complexes were eluted with sample buffer [0.125 M Tris-HCl, pH 6.8, 20% (w/v) glycerol, 4% (w/v) SDS, and 10% (v/v) β-mercaptoethanol], boiled for 3 min, then recovered by centrifugation for 5 min in a microfuge. The eluates were resolved by electrophoresis on 4-20% SDS-polyacrylamide gels. The gels were then immunoblotted with the anti-phosphotyrosine antibody 4G10 to detect the tyrosine phosphorylation of Trks or with the anti-TrkB monoclonal antibody to quantify TrkB receptor expression.

Cells were washed once with ice-cold TBS, lysed in a buffer containing 1% SDS, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 10 mM Tris-HCl pH 7.5, and 1 mM PMSF to detect the tyrosine phosphorylation of MAP kinases, and boiled for 5 min. The lysates were clarified by centrifugation at 10,000× *g* at 4°C for 1 h. The protein concentration was determined using the BCA protein assay, then 20 μg of protein was resolved by electrophoresis on 4-20% SDS-polyacrylamide gels.

Proteins were transferred onto nitrocellulose membranes in 0.1 M Tris base, 0.192 M glycine, and 20% (v/v) methanol using a semi-dry electrophoretic transfer system. The membranes were blocked with 5% (w/v) nonfat dried milk and 0.1% Tween 20 in TBS at room temperature for at least 1 h, washed three times in 0.1% Tween 20/TBS (T-TBS), then incubated with the anti-phosphotyrosine antibody 4G10 diluted to 1 : 1,000, the anti-phosphorylated MAP kinase antibody diluted to 1 : 1,000, or the anti-TrkB monoclonal antibody diluted to 1 : 5,000 in T-TBS at room temperature for 1-2 h. After three washes with T-TBS, the membranes were incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG secondary antibody (Amersham) diluted to 1 : 1,000 with T-TBS at room temperature for 1 h. The membranes were then washed four times with T-TBS, and visualized using the ECL chemiluminescence system (Amersham). Densitometry was performed using MCID (Fuji Film, Tokyo, and Imaging Research, Canada) if necessary.

RESULTS

Transfection of PC12h Cells with trkB cDNA—PC12h cells transfected with rat *trk*B cDNA were selected in the presence of G418. About 400 clones resistant to G418 were isolated and examined to determine whether they could extend neurites in response to BDNF. Two stable clones (PC-pAB1 and PC-pAB2) were selected and used in further experiments. As a control, we obtained the PC-pA cell transfected with the pAGE123 vector alone. We confirmed the expression of TrkB protein in PC-pAB1 and PC-pAB2 cells by Western blotting analysis using a monoclonal antibody specific to TrkB (Fig. 1). Total cell lysates or immunoprecipitates with the anti-pan-Trk antibody were subjected to SDS-PAGE. The TrkB protein was observed in PC-pAB1 and PC-pAB2 cells and in the cortex from rats, but not in control PC-pA cells or parental PC12h cells. Immunoprecipitation experiments with the anti-pan-Trk antibody indicated that the level of TrkB protein was 1.5-fold higher in PC-pAB1 cells than PC-pAB2 cells.

Neuronal Differentiation Induced by BDNF and NT-3—PC12 cells differentiate into neuron-like cells in response to NGF, and PC12 cells treated with NGF show marked cellular changes including neurite extension and increased acetylcholinesterase (AChE) activity. First, we compared the effects of BDNF and NGF on neurite extension of PC-pAB1 and PC-pAB2 cells. Neurites of both cell lines treated with BDNF for three days were longer than those of cells treated with NGF (Fig. 2). Neurites were observed in almost all PC-pAB1 and PC-pAB2 cells 24 h after addition of BDNF, but few cells had neurites 24 h after addition of NGF (data not shown). The rate of BDNF-induced neurite extension in PC-pAB1 cells was faster than that in PC-pAB2 cells. We also found that neurite extension was induced by NT-3 in PC-pAB1 and PC-pAB2 cells, although neurites in PC-pAB2 cells treated with NT-3 were shorter than those in PC-pAB1 cells (Fig. 2). In contrast, the control PC-pA cells extended neurites only in the presence of NGF.

Next, we examined the effects of BDNF on the activity of AChE. In PC-pAB1 cells, the activity of AChE after

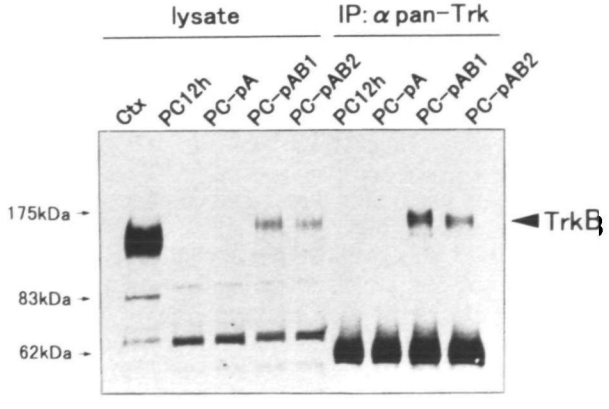

Fig. 1. Expression of TrkB protein in PC-pAB1 and PC-pAB2 cells. Total cell lysates (lysates) or immunoprecipitates with the anti-pan-Trk antibody (IP: αpan-Trk) were subjected to Western blotting analysis using the anti-TrkB monoclonal antibody. Ctx: the cortex from rats. The position of TrkB is indicated on the right of the immunoblot. The molecular weights are shown on the left.

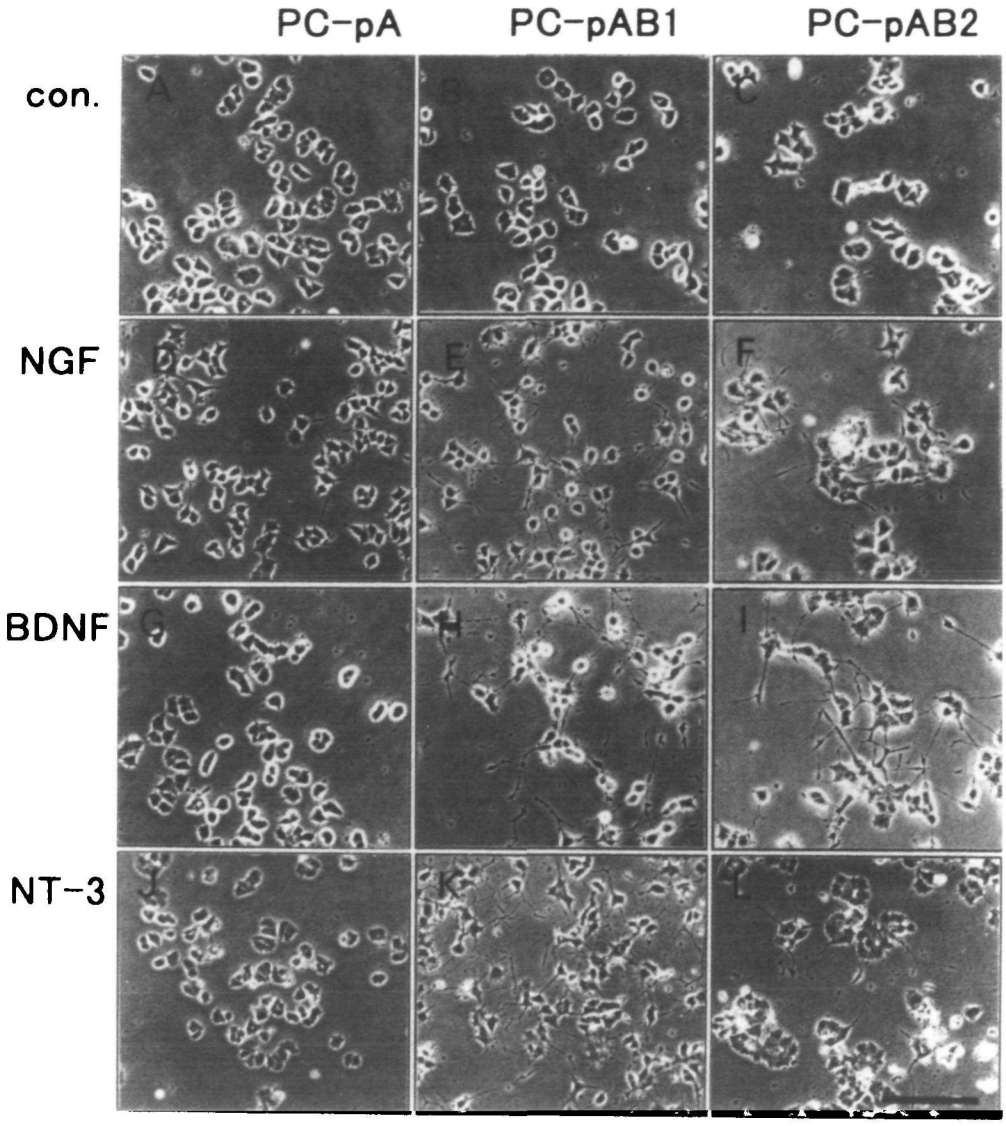

Fig. 2. **Neuronal differentiation of PC-pAB1 and PC-pAB2 cells induced by BDNF and NT-3.** PC-pA cells (A, D, G, J), PC-pAB1 cells (B, E, H, K), or PC-pAB2 cells (C, F, I, L) were cultured in DMEM supplemented with 5 μg/ml human transferrin, 5 μg/ml bovine insulin, and 20 nM progesterone in the absence (A, B, C) or presence of 50 ng/ml NGF (D, E, F), 50 ng/ml BDNF (G, H, I), or 50 ng/ml NT-3 (J, K, L) for 3 days. Photographs were taken under phase-contrast microscopy. Bar represents 50 μm.

treatment with 50 ng/ml BDNF was *ca.* 2.5-fold that of untreated cells, whereas the AChE activity after treatment with 50 ng/ml NGF was increased to *ca.* 2.8-fold (Fig. 3A). In PC-pAB2 cells, the activity of AChE after BDNF (50 ng/ml) treatment was *ca.* 1.9-fold that of untreated cells, whereas the activity after NGF (50 ng/ml) treatment was increased to *ca.* 2.2-fold (Fig. 3B). Thus, in both cell lines, NGF induced a greater increase in AChE activity than BDNF, even though it induced neurite extension more slowly than BDNF (Fig. 2). The effects of BDNF were dose-dependent, similarly to those of NGF. EC_{50} for BDNF was 1 to 10 ng/ml, which was consistent with the value reported by Ip *et al.* (*4*) for the growth response in TrkB-expressing NIH-3T3 cells and neurite outgrowth in TrkB-expressing PC12 cells. Therefore, the effect of BDNF was considered to be transduced through binding to the TrkB receptor.

Prevention of Apoptosis Induced by a High Oxygen Atmosphere or NGF Deprivation—NGF is known to protect PC12 cells from apoptotic cell death induced by various stresses. Therefore, we tested whether BDNF and NT-3 could protect PC-pAB1 and PC-pAB2 cells from cell death induced by oxygen toxicity or NGF deprivation. These types of cell death are also known to be mediated by apoptosis and are prevented by NGF in PC12 cells. Naive PC-pAB1 and PC-pAB2 cells were cultured for 2.5 days under a 50% oxygen atmosphere, and the number of viable cells was estimated by MTT assay. As shown in Fig. 4 (A, B), BDNF prevented cell death (*ca.* 120% survival) induced by oxygen toxicity in both PC-pAB1 and PC-pAB2 cells similarly to NGF (100% survival). NGF and BDNF had additive effects in preventing oxygen toxicity-induced cell death. NT-3 also prevented oxygen toxicity-induced cell death in PC-pAB1 (75% survival) and PC-pAB2 cells (90% survival) at a concentration of 50 ng/ml, at variance with the data of Ip *et al.* (*4*) who used 1,000 ng/ml NT-3 to observe similar survival-promoting effects.

To examine the survival-promoting effects of BDNF or NT-3 on death of differentiated cells on NGF deprivation, PC-pAB1 and PC-pAB2 cells were cultured for 1 week in the presence of NGF to allow differentiation into postmitotic neuron-like cells, then the medium was changed to

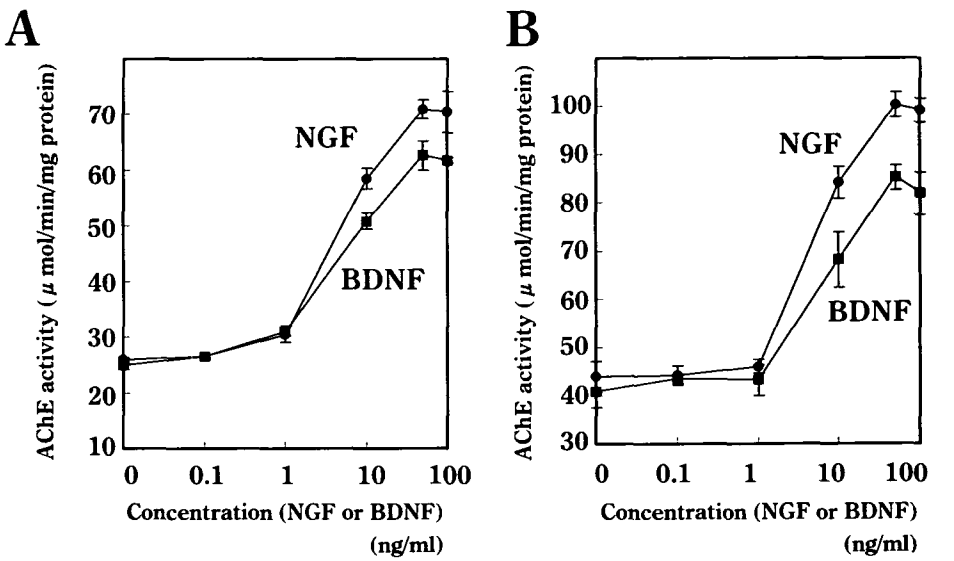

Fig. 3. **Effects of NGF and BDNF on the activity of acetylcholinesterase (AChE).** Activities of AChE in PC-pAB1 cells (A) and PC-pAB2 cells (B) treated with NGF or BDNF at indicated concentrations for 3 days were determined.

that without NGF, and cells were cultured for 4 days in the presence or absence of BDNF or NT-3. As shown in Fig. 4 (C, D), BDNF prevented cell death similarly to NGF, but NT-3 did not. These results suggest that NT-3 has a survival-promoting effect on naive PC-pAB1 and PC-pAB2 cells, but not on differentiated cells. Therefore, the survival-promoting effect of NT-3 seemed to disappear during neuronal differentiation induced by NGF. In addition, NGF and BDNF had no additive effect on prevention of cell death induced by NGF deprivation [Fig. 4 (C, D)].

Phosphorylation of TrkB and MAP Kinases in Naive PC-pAB1 and PC-pAB2 Cells—Ligand binding to the Trk receptor induces its dimerization and auto-phosphorylation on tyrosine residues, and this process is the first step in the mechanism of action of neurotrophins. To examine the tyrosine phosphorylation of TrkB in PC-pAB1 and PC-pAB2 cells, lysates of BDNF or NT-3-treated cells were immunoprecipitated with anti-pan-Trk antibody and analyzed by Western blotting with anti-phosphotyrosine antibody (4G10). In naive PC-pAB1 and PC-pAB2 cells, BDNF and NT-3 induced tyrosine phosphorylation of TrkB, whereas NGF induced tyrosine phosphorylation of TrkA (Fig. 5A). Densitometric analysis showed that the phosphorylation of TrkB by BDNF was stronger than that of TrkA by NGF in both cells. NT-3-induced tyrosine phosphorylation of TrkB was a little weaker than that by BDNF in PC-pAB1 cells. The phosphorylation of TrkB by NT-3 in PC-pAB2 cells was very weak, and this may explain the observation that only a small population of PC-pAB2 cells extended neurites in response to NT-3.

Next, we investigated the tyrosine phosphorylation of MAP kinases after treatment with BDNF or NT-3. Signal transduction through MAP kinase is important in NGF-induced differentiation of PC12 cells. The lysates prepared from naive PC-pAB1 and PC-pAB2 cells stimulated by BDNF or NT-3 were subjected to SDS-PAGE and immunoblotted with the anti-phosphotyrosine antibody 4G10 or the anti-phosphorylated MAP kinase antibody. The tyrosine phosphorylation of two MAP kinases, Erk1 and Erk2, was observed 5 min after treatment of naive PC-pAB1 and PC-pAB2 cells with NGF, BDNF or NGF+

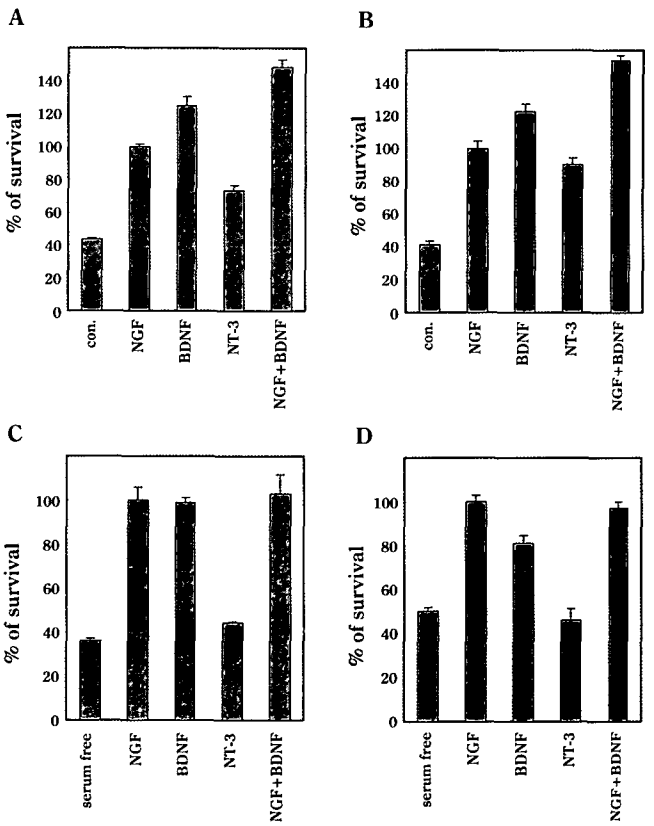

Fig. 4. **Survival-promoting effects of BDNF and NT-3 on naive and differentiated PC-pAB1 and PC-pAB2 cells.** A, B: PC-pAB1 cells (A) or PC-pAB2 cells (B) were cultured for 2.5 days under a 50% oxygen atmosphere without (con.) or with 50 ng/ml NGF, 50 ng/ml BDNF, 50 ng/ml NT-3, or 50 ng/ml NGF+50 ng/ml BDNF. The number of viable cells was estimated by MTT assay. The values are shown as percentages of the value in NGF-treated cells (100%). C, D: PC-pAB1 cells (C) or PC-pAB2 cells (D) were cultured for 7 days in the presence of NGF, then for 4 days without (serum-free) or with 50 ng/ml NGF, 50 ng/ml BDNF, 50 ng/ml NT-3, or 50 ng/ml NGF+50 ng/ml BDNF. The number of viable cells was estimated by MTT assay. The values are shown as percentages of the value in cells cultured for 4 days in the presence of NGF (100%). The values represent the means±SD of four individual cultures.

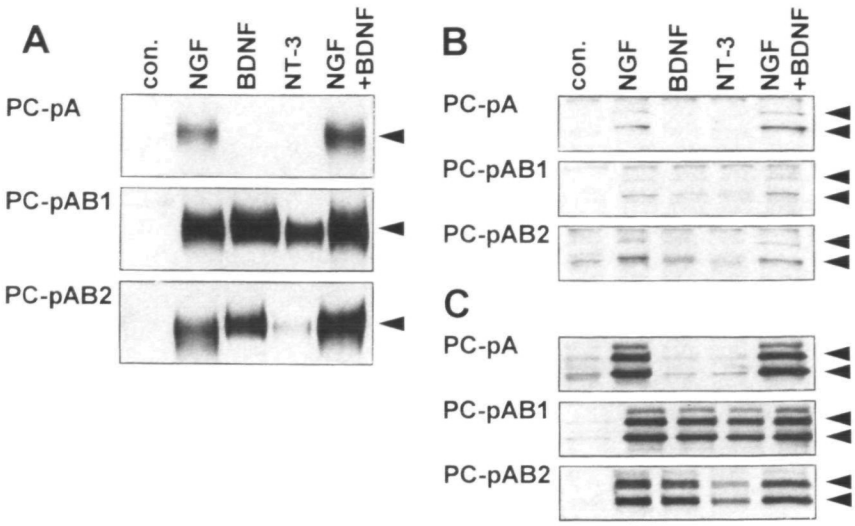

Fig. 5. **Phosphorylation of TrkB and MAP kinases in naive PC-pAB1 and PC-pAB2 cells.** Lysates were prepared from PC-pA cells, PC-pAB1 cells, or PC-pAB2 cells incubated without (con.) or with 50 ng/ml NGF, 50 ng/ml BDNF, 50 ng/ml NT-3, or 50 ng/ml NGF+50 ng/ml BDNF for 5 min. A: The lysates were immunoprecipitated with the anti-pan-Trk antibody and analyzed by Western blotting with anti-phosphotyrosine antibody. The position of Trks is indicated by a filled arrowhead. B, C: The lysates were analyzed by Western blotting with anti-phosphotyrosine antibody (B) or with anti-phosphorylated MAP kinase antibody (C). The positions of MAP kinases (Erk1 and 2) are indicated by filled arrowheads.

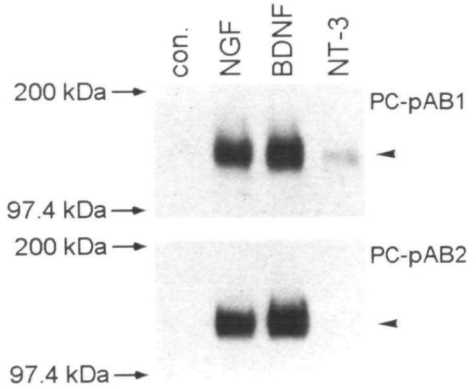

Fig. 6. **Phosphorylation of TrkB in differentiated PC-pAB1 and PC-pAB2 cells.** PC-pAB1 cells or PC-pAB2 cells were cultured for 7 days in TIP/DF medium containing 50 ng/ml NGF. Differentiated PC-pAB1 cells or PC-pAB2 cells were placed with DMEM 2 h before addition of neurotrophin. Lysates were prepared from differentiated PC-pAB1 cells or PC-pAB2 cells incubated without (con.) or with 50 ng/ml NGF, 50 ng/ml BDNF, or 50 ng/ml NT-3 for 5 min. The lysates were immunoprecipitated with anti-pan-Trk antibody and analyzed by Western blotting with anti-phosphotyrosine antibody. The position of Trks is indicated by a filled arrowhead.

BDNF (Fig. 5B). The phosphorylation of MAP kinases induced by NGF was stronger than that by BDNF in both cell lines, even though the phosphorylation of TrkA by NGF was weaker than that of TrkB by BDNF. NT-3 induced very little tyrosine phosphorylation of MAP kinases in PC-pAB1 cells, and none was detected in PC-pAB2 cells. However, in the experiment with the anti-phosphorylated MAP kinase antibody, which is more sensitive than the anti-phosphotyrosine antibody 4G10, we observed the tyrosine phosphorylation of MAP kinases 5 min after addition of NT-3 in both cell lines (Fig. 5C).

Phosphorylation of TrkB in Differentiated PC-pAB1 and PC-pAB2 Cells—To investigate why NT-3 did not have a survival-promoting effect on differentiated PC-pAB1 and PC-pAB2 cells, we examined the tyrosine phosphorylation of TrkB in differentiated cells treated with BDNF or NT-3. As shown in Fig. 6, BDNF induced the tyrosine phosphorylation of TrkB in both differentiated cell lines, but NT-3 did not induce it in either one. These results indicate that NT-3 cannot induce the tyrosine phosphorylation of TrkB, and as a result it cannot prevent apoptosis induced by NGF deprivation in differentiated PC-pAB1 and PC-pAB2 cells.

DISCUSSION

To isolate PC12 cell lines stably expressing the TrkB receptor (*4, 25*), we transfected PC12h cells with recombinant pAGE123 containing the full-length rat *trk*B cDNA using LipofectAMINE. The pAGE123 vector contains the Rous sarcoma virus long terminal repeat (RSV LTR) as a promoter. We compared pAGE123 with another vector, pRc/RSV, which also contains RSV LTR. With the pRc/RSV vector we could not obtain PC12h cell lines stably expressing TrkB (data not shown). The expression of *trk*B cDNA in two obtained cell lines (PC-pAB1 and PC-pAB2) was confirmed by Northern blotting (data not shown) and Western blotting analyses. The level of TrkB protein was 1.5-fold higher in PC-pAB1 cells than PC-pAB2 cells (Fig. 1). The difference in the protein level between the two cell lines may explain the differences in their responses to BDNF or NT-3.

The rate of neurite extension induced by BDNF was faster in PC-pAB1 cells than PC-pAB2 cells (Fig. 2). It was reported that overexpression of TrkA accelerated NGF-induced neurite extension in PC12 cells (*26*). These results indicate that the rate of neurite extension induced by these neurotrophins is dependent on the level of their receptors. In this context, it was reasonable that BDNF could induce neurite outgrowth more rapidly than NGF in both PC-pAB1 and PC-pAB2 cells (Fig. 2). On the other hand, the activity of AChE after treatment with NGF was higher than that with BDNF in both PC-pAB1 and PC-pAB2 cells (Fig. 3). We consider that the signaling pathways leading to neurite extension and to the activation of AChE are not the same and the latter is not dependent on the level of Trk receptor protein.

We observed neurite extension in TrkB-expressing PC12h cells induced by NT-3, which is the cognate ligand of the TrkC receptor (Fig. 2). It was reported that NT-3 could induce growth responses in TrkB-expressing NIH3T3 cells and neurite extension in TrkB-expressing PC12 cells (*4, 27*). Strohmaier *et al.* (*28*) have recently reported that there are two splice variants of *trk*B mRNA in the chicken, *ctrk*B-L and *ctrk*B-S. *ctrk*B-L encodes cTrkB-L, which is 11

amino acids longer than cTrkB-S encoded by *ctrk*B-S, and cTrkB-L can respond to NT-3 more efficiently than cTrkB-S. We consider that the rat *trk*B cDNA used in our studies corresponds to *ctrk*B-L and therefore the TrkB-expressing PC12h cells could respond to NT-3 efficiently. The fact that neurites in PC-pAB2 cells treated with NT-3 were weaker than those in PC-pAB1 cells (Fig. 2) was consistent with the observation that the tyrosine phosphorylation of TrkB and MAP kinases induced by NT-3 in PC-pAB2 cells was weaker than that in PC-pAB1 cells (Fig. 5).

Apoptosis of neuronal cells induced by NGF and/or serum deprivation is considered to be a model of physiological neuronal cell death, and apoptosis of neuronal cells induced by oxygen toxicity is a model of pathological neuronal cell death. We found that BDNF could protect TrkB-expressing PC12h cells from apoptosis induced by NGF deprivation and oxygen toxicity (Fig. 4). BDNF also protects cerebellar granule neurons from low-K^+-induced apoptosis (*14, 15*). These results indicate that BDNF, as well as NGF, can activate a survival-promoting pathway among intracellular signal transduction pathways. The survival-promoting effect of NGF on apoptosis induced by NGF deprivation and that of BDNF on low K^+-induced apoptosis were blocked by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase (PI3-K), indicating that PI3-K is involved in the survival pathway induced by neurotrophins (*11, 15, 29*). However, the signaling pathway downstream of PI3-K is unclear at present. Some molecules such as p70 ribosomal protein kinase ($p70^{S6K}$), serine-threonine kinase Akt and calcium-insensitive types of protein kinase C (PKC) can be activated by PI3-K (*30-33*). We found that $p70^{S6K}$ is not involved in the BDNF-mediated survival pathway in cerebellar granule neurons (*15*). On the other hand, Zirrgiebel *et al.* (*34*) suggested that BDNF promotes the survival of cerebellar granule neurons *via* PKC activation. More recently, Dudek *et al.* (*35*) have reported that Akt is a critical mediator of growth factor-induced neuronal survival. Further analyses at the molecular level are required to determine how Akt and/or PKC are involved in the survival pathway.

The observation that BDNF and NGF could protect TrkB-expressing cells to similar levels from apoptosis induced by oxygen toxicity and NGF deprivation (Fig. 4) suggests that the survival pathway is efficiently transduced *via* ordinary levels of the receptor and is not enhanced by overexpression of TrkB receptor. As shown in Fig. 4 (A, B), an additive effect of NGF and BDNF was observed in prevention of apoptosis induced by oxygen toxicity in naive cells. However, the apparent additivity is considered to be due to the additivity in transient induction of the growth response by NGF and BDNF (data not shown). In fact, there was no additive effect of NGF and BDNF in prevention of apoptosis induced by NGF deprivation in differentiated cells [Fig. 4 (C, D)].

Although the differentiation-inducing effect of NT-3 was considerably weaker than those of NGF and BDNF (Fig. 2), the survival-promoting effect of NT-3 was a little weaker than those of NGF and BDNF in naive TrkB-expressing cells [Fig. 4 (A, B)]. These results suggest that the survival pathway can be transduced by weak activation of TrkB by NT-3.

As shown in Fig. 4 (C, D), NT-3 did not prevent apoptosis induced by NGF deprivation in differentiated TrkB-expressing cells, indicating that the survival-promoting effect of NT-3 disappears during neuronal differentiation. Ip *et al.* (*4*) reported that the interaction of NT-3 with TrkB is weaker in naive PC12 cells than in NIH3T3 cells. We further compared NT-3–TrkB interactions between naive PC12 cells and differentiated PC12 cells, and found that NT-3 could not act on TrkB in neuronally differentiated PC12 cells (Fig. 6). This might reflect a similar inability of NT-3 to interact with TrkB in PNS and CNS neurons. We are planning to search for accessory molecules of TrkB which might determine the ligand selectivity and be induced during neuronal differentiation in our TrkB-expressing PC12h cells.

We compared the tyrosine phosphorylation of Trk receptors and MAP kinases induced by NGF, BDNF, or NT-3 in naive TrkB-expressing cells (Fig. 5). Although the tyrosine phosphorylation of TrkA by NGF was weaker than that of TrkB by BDNF, the phosphorylation of MAP kinases induced by NGF was stronger than that by BDNF in the two TrkB-expressing cell lines examined. Both TrkA and TrkB have the tyrosine residues which can mediate the interaction with Shc (*2, 10*). The level of tyrosine phosphorylation of Shc might be different between TrkA and TrkB. Although the tyrosine phosphorylation of MAP kinases induced by BDNF was weaker than that by NGF, BDNF induced neurite extension more rapidly than NGF in both TrkB-expressing cell lines. These results suggest that TrkB activates signaling pathways other than the Ras-MAP kinase pathway, leading to neuronal differentiation. Candidates for such signaling pathways include SNT (*36*), cAMP-dependent protein kinase (PKA) (*37-39*), and STAT (*40-42*).

The experiments of Fig. 5B, in which we used the anti-phosphotyrosine antibody 4G10, showed that little or no tyrosine phosphorylation of MAP kinases was induced by NT-3 in two naive TrkB-expressing cells. But in the experiments with the anti-phosphorylated MAP kinase antibody, we observed the tyrosine phosphorylation of MAP kinases after addition of NT-3 in both cell lines (Fig. 5C). These results indicate that the anti-phosphorylated MAP kinase antibody can be used to detect low levels of tyrosine phosphorylation of MAP kinases.

Further studies are necessary to elucidate the detailed molecular mechanisms involved in the neurotrophin-induced signaling pathways which lead to neuronal survival.

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