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Early BDNF, NT-3, and NT-4 Signaling Events

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Much more is known about nerve growth factor (NGF) signaling than that initiated by brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), or NT-4. We sought to study early BDNF, NT-3, and NT-4 signaling events. Using TrkB-expressing cells, we found that BDNF and NT-4 individually induced tyrosine phosphorylation of TrkB in a dose-dependent fashion. At maximally effective concentrations, BDNF or NT-4 induced robust TrkB tyrosine phosphorylation at 5 min; this progressively declined at 15, 30, and 60 min. Using immunoprecipitation, PI3-kinase and tyrosine phosphorylated PLC-γ1 and SHC were shown to be associated with tyrosine phosphorylated TrkB in response to both BDNF and NT-4. BDNF and NT-4 induced similar intensities of phosphorylation of TrkB and signaling intermediates at equivalent doses. NT-3 treatment of TrkC-expressing cells induced very similar patterns for induction of TrkC tyrosine phosphorylation and recruitment of signaling intermediates. BDNF, NT-3, and NT-4 caused rapid tyrosine phosphorylation of ERK and SNT. These data suggest that the earliest signaling events for BDNF, NT-3, and NT-4 are very similar to those for NGF. © 1999 Academic Press

Key Words: **neurotrophin signaling; brain-derived neurotrophic factor; neurotrophin-3; neurotrophin-4; Trk receptor; TrkB; TrkC.**

INTRODUCTION

Neurotrophic factors are growth factors that act directly on neurons to support their growth, differentiation, and survival (57). The neurotrophin family of neurotrophic factors includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4 (4). They signal through the Trk family of receptor tyrosine kinases. NGF signals primarily through TrkA, BDNF and NT-4 through TrkB, and NT-3 through TrkC. All of the neurotrophins bind to another receptor, $p75^{NTR}$, whose signaling function is less well characterized. Studies of $p75^{NTR}$ signaling have suggested that it signals on its own as well as cooperating with the Trks (8). However, $p75^{NTR}$ is neither necessary nor sufficient for many of the known neurotrophin functions.

NGF was the first neurotrophic factor discovered (36). Much has been learned about NGF function and signaling since its discovery in the 1950s (reviewed in (4) and (26)). NGF signaling through TrkA involves tyrosine phosphorylation of TrkA and subsequent association of the activated receptor with the phosphorylated signaling intermediates phospholipase C-γ1 (PLC-γ1), SHC, and phosphatidylinositol 3-kinase (PI3-kinase). Other downstream effects of TrkA activation include tyrosine phosphorylation of the signaling intermediates ERK and SNT.

Brain-derived neurotrophic factor (BDNF) was the second neurotrophin discovered (38). NT-3 and NT-4 were discovered in the early 1990s based upon DNA sequences similar to NGF and BDNF (4). The signaling pathways of BDNF, NT-3, and NT-4 are much less well characterized compared to NGF. *In vitro* and *in vivo* studies comparing BDNF and NT-4 have shown both similar (1, 6, 9, 10, 13, 15–17, 20, 21, 23, 28, 32, 33, 35, 52, 54, 55, 58, 59) and different (2, 11, 12, 22, 29, 37, 49, 50, 53, 56) effects depending upon the experimental paradigm. It is at present unclear whether BDNF and NT-4 signaling through TrkB involves the same signaling intermediates.

In this study, we used NIH 3T3 cells (murine fibroblasts) stably transfected with full-length, functional rat TrkB or TrkC to study BDNF, NT-4, and NT-3 signaling (18, 24). These cells provided a means to study TrkB and TrkC signaling in a common cellular context.

MATERIALS AND METHODS

Materials. NGF was prepared as described (61). Recombinant human BDNF and NT-3 were generously provided by Regeneron Pharmaceuticals (Tarrytown, PA). NT-4 was purchased from Promega (Madison, WI). 1088 is a rabbit antibody against the C-terminus of human TrkA; it was purified using Protein A–Sepharose (Pierce, Rockford, IL) and has been characterized previously (61). Sc11 is another rabbit antibody to the C-terminus of human TrkA (Santa Cruz Biotechnology,

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Santa Cruz, CA). Except as noted, all other reagents and chemicals were purchased from Sigma (St. Louis, MO).

Cell culture. NIH 3T3 cells (designated as MG86/87 by Regeneron Pharmaceuticals) stably transfected with TrkB or TrkC individually were provided by Regeneron Pharmaceuticals and have been described previously (18, 24). These cells do not express $p75^{NTR}$. Cells were grown on plastic culture plates (Falcon) in Dulbecco's modified Eagle's medium (DME-H21) supplemented with 10% bovine calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 400 µg/ml G418.

Neurotrophin treatment. For experiments with neurotrophin treatment, the appropriate concentration of neurotrophin was added to a culture plate with cells at 50–70% confluency. Cells were incubated at 37°C with the neurotrophin or vehicle (phosphate-buffered saline; PBS) for a predetermined amount of time. The reaction was then stopped by washing the cells once with ice-cold PBS. Cells were then lysed by the addition of 1 ml of lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet-P40, 10% glycerol, 1 mM phenlymethylsulfonyl fluoride, 10 µg/ml aprotinin, 1 µg/ml leupeptin, and 500 µM sodium orthovanadate). After centrifugation at 10,000*g* for 10 min, the supernatant was removed and assayed for protein concentration (BCA Assay; Pierce).

Immunoprecipitation and western blotting. Cell lysates were normalized for protein concentration and volume and immunoprecipitated with a specific antibody, rotating overnight at 4°C. Antibodies that were used included 12 µg/ml 1088, 6 µg/ml anti-PLC-γ1 (Upstate Biotechnology, Lake Placid, NY), 6 µg/ml anti-SHC (Upstate Biotechnology), 6 µg/ml anti-PI3-kinase (p85 subunit) (Upstate Biotechnology and Santa Cruz Biotechnology), and 2 µg/ml anti-ERK1 (Santa Cruz Biotechnology). When examining for SNT, 25 µl/ml $p13^{suc1}$-agarose (Upstate Biotechnology) was used for affinity purification. To Protein A–Sepharose beads (Pierce), 100 µl of a 25% solution per milliliter of lysate was added and incubated at 4°C for 2 h. After being washed twice with lysis buffer and once with H_2O, 50 µl of 2× sample buffer (100 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.2% Bromphenol blue, and 200 mM DTT) was added to the pellet and the sample was heated to 100°C for 5 min. Samples were run on 7.5% SDS–PAGE and then transferred to nitrocellulose. Blots were probed with PY20 (anti-phosphotyrosine antibody; Transduction Laboratories, Lexington, KY), Sc11, or the immunoprecipitating antibody. Immune complexes were detected with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG, chemiluminescence (Amersham, Arlington Heights, IL), and XAR-5 film (Eastman Kodak, Rochester, NY). Blot reprobes were performed by stripping with 20 mM Tris, pH 2.0, 137 mM NaCl for 10 min and then probing and developing as described above. Data were quantified from multiple chemiluminescent exposures using NIH Image.

RESULTS

Dose-Response of Neurotrophin-Induced Phosphorylation of TrkB or TrkC

We used NIH 3T3 cells (murine fibroblasts) stably transfected with full-length, functional rat TrkB or TrkC to study BDNF, NT-4, and NT-3 signaling. These cell lines expressed similar amounts of TrkB and TrkC protein based upon Western blot analysis of the intensity of Trk protein bands (Yuen and Mobley, unpublished observation). We sought to determine the dose-response for neurotrophin treatment and TrkB or TrkC tyrosine phosphorylation. TrkB-transfected NIH 3T3 cells were incubated with increasing concentrations of BDNF or NT-4, and TrkC-transfected cells were incubated with NT-3. Incubations were for 5 min at 37°C, and this was followed by immunoprecipitation of Trk and detection by anti-phosphotyrosine antibody following Western blotting (Fig. 1). There was no appreciable tyrosine phosphorylation of TrkB or TrkC when cells were not treated with BDNF, NT-4, or NT-3. There were progressive increases in TrkB phosphorylation intensities with increasing concentrations of BDNF or NT-4, and the maximum signal was activated with 200 ng/ml. The dose–response curves were virtually identical for BDNF and NT-4 (Fig. 1D). In contrast, the dose–response curve for TrkC phosphorylation in response to NT-3 was shifted to the left, and TrkC phosphorylation was maximal at 50 ng/ml of NT-3. The ED_{50} (dose at which Trk was phosphorylated at 50% of maximum) for TrkC phosphorylation was much lower for NT-3 (5 ng/ml) than the ED_{50} for TrkB treated with BDNF or NT-4 (50 ng/ml). Untransfected parental NIH 3T3 cells did not exhibit TrkB or TrkC tyrosine phosphorylation in response to BDNF or NT-3 treatment (data not shown).

Time Course of Neurotrophin-Induced Phosphorylation of TrkB or TrkC

In PC12 (pheochromocytoma) cells, NGF treatment results in TrkA tyrosine phosphorylation that peaks at 5 min of treatment and is reduced over the next hour due, in part, to internalization and degradation of the receptor (61). To determine whether TrkB or TrkC tyrosine phosphorylation is similarly reduced, we examined the time course of Trk tyrosine phosphorylation in response to neurotrophins at the concentration required to produce the maximum response in the dose–response studies. TrkB-transfected NIH 3T3 cells were incubated with BDNF or NT-4, and TrkC-transfected cells were incubated with NT-3 for various lengths of time at 37°C, followed by immunoprecipitation of Trk

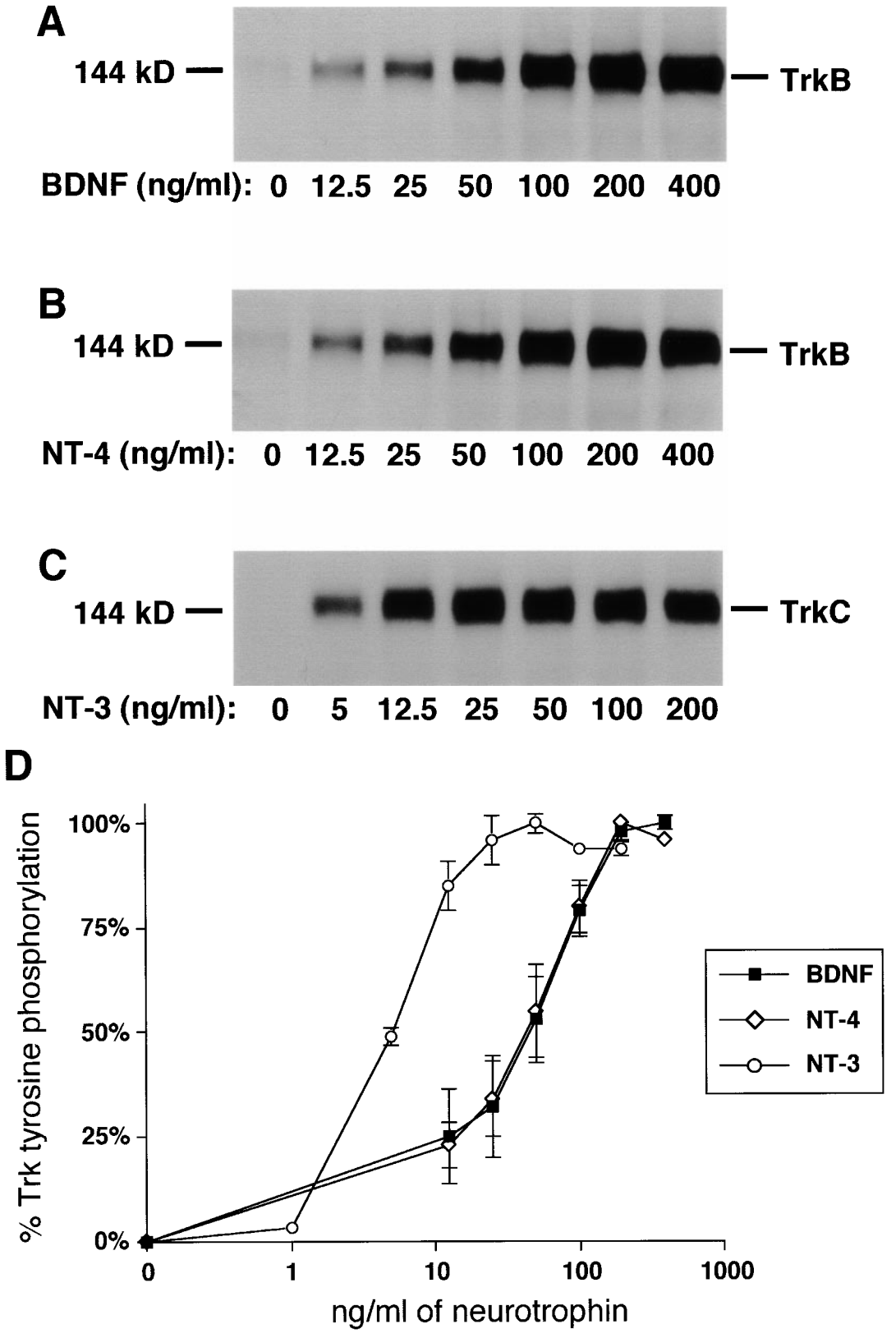


FIG. 1. TrkB or TrkC tyrosine phosphorylation increased with increasing concentrations of BDNF (A), NT-4 (B), or NT-3 (C) treatment. The dose–response curves are plotted (D). Equal numbers of TrkB-expressing 3T3 cells were incubated with various concentrations of BDNF or NT-4 for 5 min at 37°C. The cells were washed once with ice-cold PBS and then lysed. Equal protein concentrations and volumes of cell lysates were immunoprecipitated with anti-Trk antibody (1088) followed by SDS–PAGE and transfer to nitrocellulose. Blots were probed with anti-phosphotyrosine antibody. The same procedure was used with TrkC-expressing 3T3 cells and NT-3 treatment. Reprobe of blots with anti-Trk antibody (sc-11) revealed bands at the same location (data not shown).

and detection by anti-phosphotyrosine antibody following Western blotting (Fig. 2). TrkB phosphorylation was greatest at 5 min of BDNF or NT-4 treatment and decreased progressively at 15, 30, and 60 min. The intensities of TrkB phosphorylation at the various time points were similar for BDNF and NT-4 treatment. TrkC phosphorylation was also greatest at 5 min of NT-3 treatment and decreased progressively at 15, 30, and 60 min. The pattern for TrkC was similar to that for TrkB.

PLC-γ1 and SHC Tyrosine Phosphorylation and Association with Trk in Response to Neurotrophin Treatment

In TrkA-expressing cells, NGF induces tyrosine phosphorylation of TrkA, followed by association of PLC-γ1 and SHC with TrkA and tyrosine phosphorylation of these signaling intermediates (4). We examined the activation of PLC-γ1 and SHC following 5 min of treatment of TrkB- or TrkC-expressing NIH 3T3 cells with increasing doses of neurotrophin at 37°C. Cell lysates were immunoprecipitated with antibodies to PLC-γ1 or SHC. After transfer, anti-phosphotyrosine antibody was used to detect immunoprecipitated phosphorylated PLC-γ1 or SHC, and anti-Trk antibody was used to detect coprecipitated phosphorylated Trk.

PLC-γ1 was tyrosine phosphorylated in response to the maximum effective concentrations of BDNF or NT-3 acting on TrkB- or TrkC-expressing cells, respectively. PLC-γ1 phosphorylation is represented by a band at approximately 150 kDa (Fig. 3A). Immunoprecipitation of PLC-γ1 by anti-PLC-γ1 antibodies resulted in coprecipitation of phosphorylated TrkB or TrkC. Phosphorylated TrkB or TrkC associated with PLC-γ1 is visualized by the wide bands at 145 kDa following probing with anti-phosphotyrosine antibodies (Fig. 3A) or anti-Trk antibodies (Fig. 3B). The coprecipitation of TrkB or TrkC with immunoprecipitation of PLC-γ1 is evidence that PLC-γ1 and the Trk receptor associated in response to neurotrophin treatment. Note the greater intensities of PLC-γ1 and TrkB phosphorylation in response to BDNF compared to the intensities of PLC-γ1 and TrkC in response to NT-3. This finding was reproduced in three separate experiments. There was a

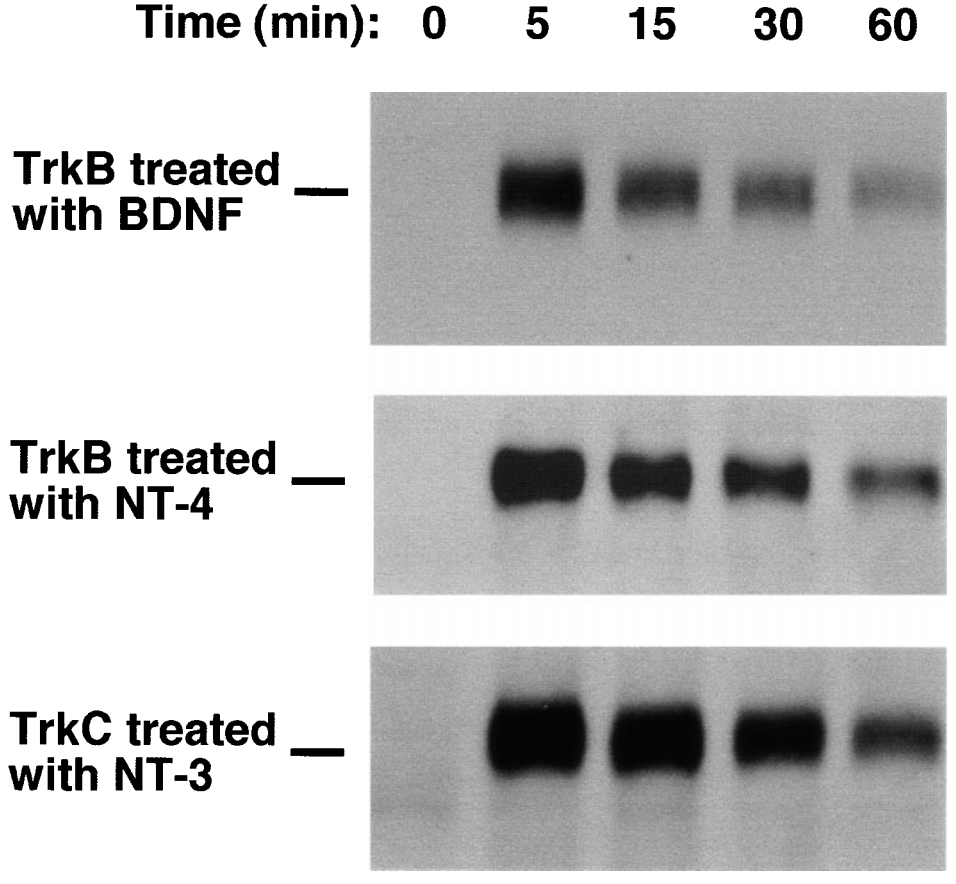


FIG. 2. TrkB or TrkC tyrosine phosphorylation peaked at 5 min of NT treatment and decreased thereafter. BDNF (200 ng/ml; A), NT-4 (200 ng/ml; B), and NT-3 (50 ng/ml; C). TrkB- or TrkC-expressing 3T3 cells were treated with NT, lysed, and then immunoprecipitated with anti-Trk antibody after normalization of protein concentrations and volumes. Blots were probed with anti-phosphotyrosine antibody. Reprobe of blots with anti-Trk antibody (sc-11) revealed bands at the same location (data not shown).

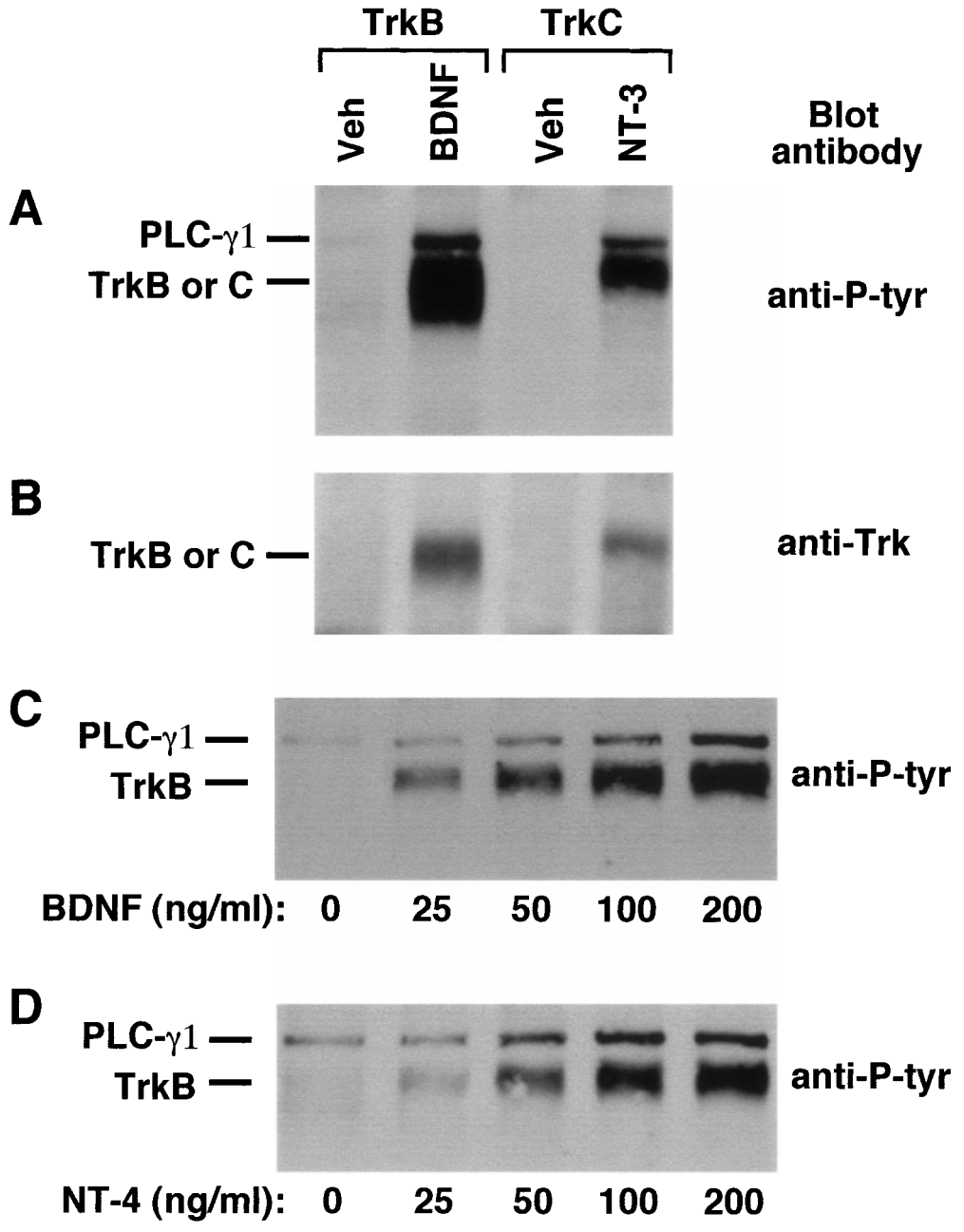


FIG. 3. PLC-γ1 was tyrosine phosphorylated and associated with tyrosine phosphorylated TrkB or TrkC in response to 5 min of treatment with BDNF 200 ng/ml (A) or NT-3 50 ng/ml (B). There was a dose-dependency for PLC-γ1 phosphorylation and association with phosphorylated TrkB with increasing BDNF (C) or NT-4 (D). There was no appreciable difference between BDNF and NT-4 induction of PLC-γ1 phosphorylation and association with phosphorylated TrkB for a given concentration of BDNF or NT-4. TrkB- or TrkC-expressing 3T3 cells were treated with NT, lysed, and then immunoprecipitated with anti-PLC-γ1 antibody. Blots were probed with anti-phosphotyrosine antibody (A, C and D) and then anti-Trk antibody (B).

dose-dependency of PLC-γ1 phosphorylation with increasing BDNF or NT-4 concentrations (Figs. 3C and 3D). They were similar to that for activation of TrkB by these ligands. Furthermore, increasing BDNF or NT-4 concentrations induced a progressively increasing degree of association with phosphorylated TrkB. There was no notable difference between the dose-dependency for BDNF and NT-4 induction of PLC-γ1 phosphorylation or for association of PLC-γ1 with phosphorylated TrkB.

Similar to what we found with PLC-γ1, SHC (48, 53, and 63 kDa) was tyrosine phosphorylated in response to treatment with maximally effective doses of BDNF or NT-3 in TrkB- or TrkC-transfected cells, respectively

(Figs. 4A and 4B). BDNF or NT-3 treatment also led to the coprecipitation of tyrosine phosphorylated TrkB or TrkC (145 kDa), respectively (Fig. 4A). Note that the intensities of SHC and TrkB phosphorylation in response to BDNF are less than for SHC and TrkC in response to NT-3. This difference was reproduced in three separate experiments. The finding was in the opposite direction to that seen for PLC-γ1. There was a dose-dependency of SHC phosphorylation with increasing BDNF or NT-4 concentrations (Figs. 4C–4F), and they were similar to those for activation of TrkB. There was no notable difference between BDNF and NT-4 induction of SHC phosphorylation for a given concentration of BDNF or NT-4.

Neurotrophin-Induced ERK Phosphorylation

Given the similarities seen in early signaling events, we examined ERK activation, a downstream marker of TrkB signaling. ERK (MAP kinase) is a serine–threonine kinase involved in NGF signaling (5). It has been shown to be activated by tyrosine phosphorylation following NGF treatment in TrkA-expressing cells (5). To examine TrkB or TrkC signaling through ERK, we treated TrkB- or TrkC-transfected cells with maximally effective doses of BDNF or NT-3, respectively. Following immunoprecipitation of ERK1, Western blotting, and detection with anti-phosphotyrosine antibody, we found that ERK1 tyrosine phosphorylation was increased, as demonstrated by a 44-kDa band (Fig. 5A). Two higher weight bands that coprecipitated with ERK1 also had increased tyrosine phosphorylation in response to neurotrophin treatment. These bands most likely represent other forms of ERK, including ERK4 (5). Note the similarities in ERK tyrosine phosphorylation intensities between TrkB- and TrkC-transfected cells. There was no appreciable difference in ERK1 tyrosine phosphorylation intensity for a given concentration of BDNF or NT-4 (Figs. 5B and 5C). Interestingly, ERK1 phosphorylation was maximal at BDNF and NT-4 concentrations of 25 ng/ml; this was much lower (approximately eightfold) than the concentrations needed to maximize TrkB, PLC-γ1, and SHC tyrosine phosphorylation.

Neurotrophin-Induced SNT Phosphorylation

A less well-defined signaling event following TrkA activation involves tyrosine phosphorylation of the signaling intermediate SNT (suc-associated neurotrophic factor-induced tyrosine-phosphorylated target) (44). The function of SNT is not well defined, but Kaplan and Stephens proposed that it is specific for NGF signaling leading to cell differentiation and is not associated with cell proliferation (27). SNT has been shown to bind to the protein $p13^{suc1}$, which interacts with $p34^{cdc2}$. Using $p13^{suc1}$ bound to agarose, we affinity-precipitated SNT (78 and 90 kDa) and found that it was tyrosine phosphorylated in response to 5 min of treatment with maximally effective concentrations of BDNF or NT-4 in TrkB-transfected cells (Fig. 6). SNT was similarly tyrosine phosphorylated in response to NT-3 treatment of TrkC-transfected cells (Fig. 6).

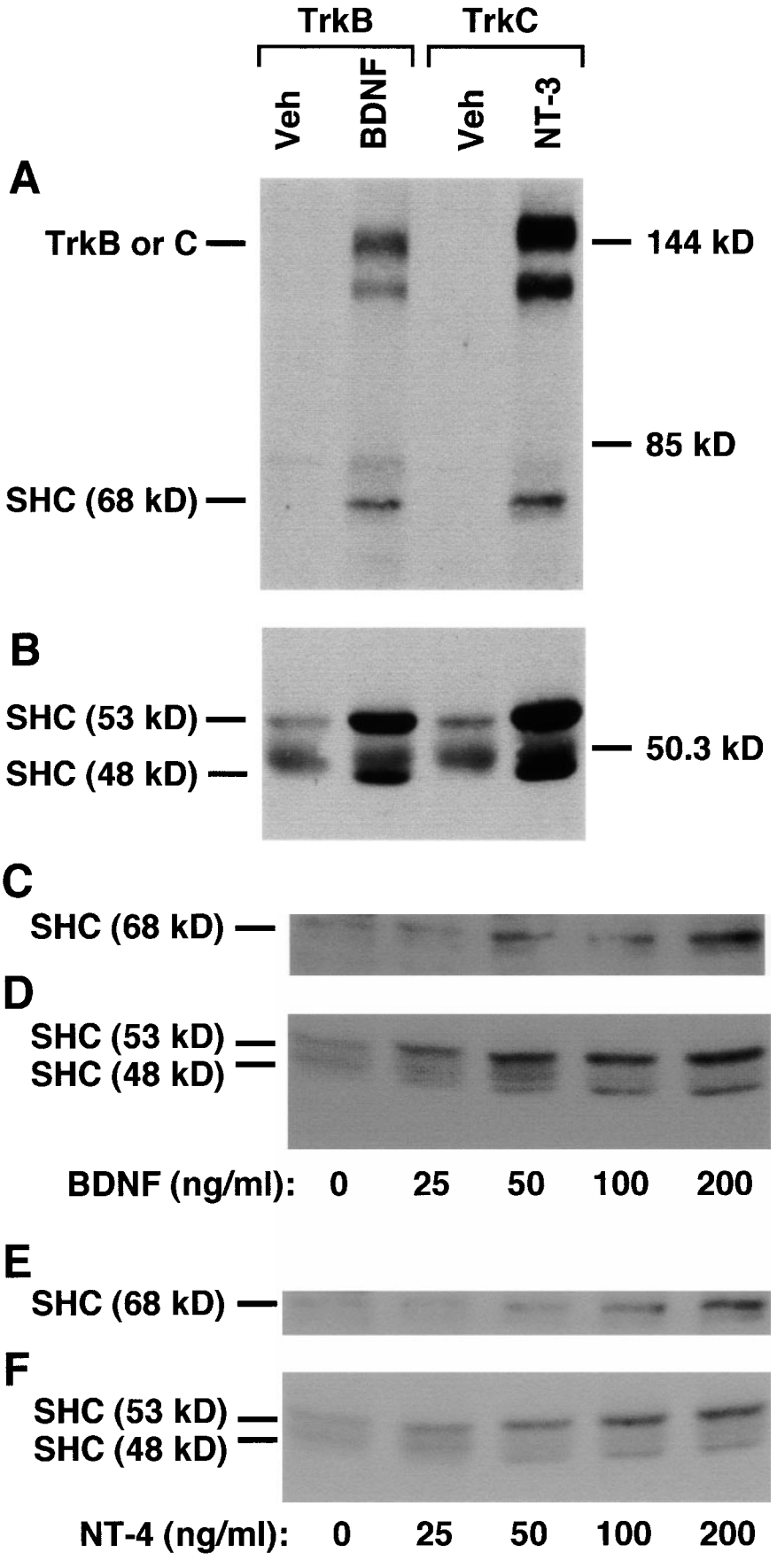
PI3-Kinase Association with Tyrosine Phosphorylated Trk in Response to Neurotrophin Treatment

There has been increasing interest in the role of PI3-kinase activation on neuronal survival. In TrkA-expressing cells, NGF treatment induces activation of PI3-kinase and its association with tyrosine phosphorylated TrkA (47). PI3-kinase activation leads to downstream signaling through Akt binding and activation (14, 26). We examined the association of PI3-kinase following 5 min of treatment of TrkB- or TrkC-expressing NIH 3T3 cells with maximally effective doses of neurotrophin at 37°C. Cell lysates were immunoprecipitated with antibodies to the p85 subunit of PI3-kinase and subjected to Western blotting. We found that tyrosine phosphorylated TrkB or TrkC was coprecipitated with PI3-kinase in response to neurotrophin treatment, as depicted by bands at 145 kDa in blots probed with anti-phosphotyrosine (Fig. 7A) and anti-Trk (Fig. 7B) antibodies. There was no convincing evidence for phosphorylation of the p85 subunit or its association with phosphorylated p110 subunit. Our findings are similar to other results demonstrating PI3-kinase activation and association with activated receptor tyrosine kinases without PI3-kinase tyrosine phosphorylation (3, 25, 40, 42, 46, 51).

DISCUSSION

Earlier studies of the signaling events induced by BDNF and NT-3 have been described by others (18, 19, 30, 31, 39, 41), but the details of the dose-dependency and the time courses of TrkB or TrkC phosphorylation and of the activation of signaling intermediates have been less thoroughly examined. We examined BDNF

FIG. 4. SHC was tyrosine phosphorylated and associated with tyrosine phosphorylated TrkB or TrkC (A and B) in response to 5 min of NT treatment at maximally effective doses. There was a dose-dependency for SHC phosphorylation and association with phosphorylated TrkB with increasing BDNF or NT-4 concentrations. There was no notable difference between BDNF and NT-4 induction of SHC phosphorylation for a given concentration of BDNF or NT-4 (C, D, E, and F). TrkB- or TrkC-expressing 3T3 cells were treated with NT, lysed, and then immunoprecipitated with anti-SHC antibody. Blots were probed with anti-phosphotyrosine antibody. Reprobe of blot A with anti-Trk antibody revealed Trk protein at levels comparable to those suggested by the amount of tyrosine phosphorylated receptor (data not shown).


TrkB
TrkC
Veh
BDNF
Veh
NT-3
A
TrkB or C
144 kD
85 kD
SHC (68 kD)
B
SHC (53 kD)
SHC (48 kD)
50.3 kD
C
SHC (68 kD)
D
SHC (53 kD)
SHC (48 kD)
BDNF (ng/ml): 0 25 50 100 200
E
SHC (68 kD)
F
SHC (53 kD)
SHC (48 kD)
NT-4 (ng/ml): 0 25 50 100 200

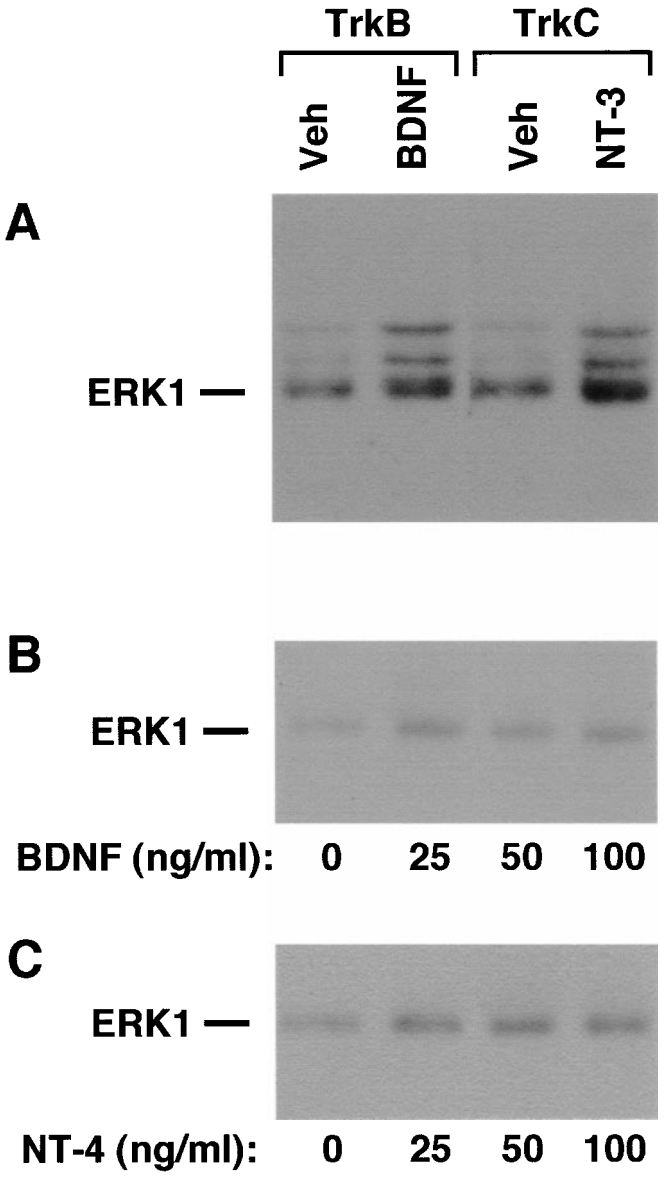


FIG. 5. ERK1 tyrosine phosphorylation was increased in response to 5 min of treatment with BDNF (200 ng/ml) or NT-3 (50 ng/ml) (A). There was no appreciable difference between BDNF and NT-4 induced ERK1 tyrosine phosphorylation with increasing doses of NT (B and C). Equal protein concentrations and volumes of cell lysates were immunoprecipitated with anti-ERK1 antibody. Blots were probed with anti-phosphotyrosine antibody.

and NT-4 signaling through TrkB and NT-3 signaling through TrkC in NIH 3T3 cells transfected with either TrkB or TrkC to provide a common cellular context for the analysis. There was increasing TrkB or TrkC tyrosine phosphorylation with increasing BDNF, NT-4, or NT-3 concentrations. At maximally effective doses, both TrkB and TrkC phosphorylation peaked after 5 min, and this was followed by decreasing Trk phosphorylation over a very similar time course. BDNF, NT-4, and NT-3 activation of Trk receptors resulted in the association of activated Trk with the signaling intermediates PI3-kinase and tyrosine phosphorylated PLC-γ1 and SHC, and each of these treatments induced tyrosine phosphorylation of ERK1 and SNT. Thus, many of the early signaling events through TrkB and TrkC appear to be quite similar.

Despite the similarities, we found certain quantitative differences between BDNF and NT-3 signaling. First, there was a clear difference in dose-dependency. BDNF maximized TrkB phosphorylation at 200 ng/ml, whereas NT-3 saturated TrkC phosphorylation at the considerably lower concentration of 50 ng/ml. The ED_{50} for BDNF was approximately 50 ng/ml, an order of magnitude greater than for NT-3. This difference may be due to higher binding affinity of NT-3 to TrkC than BDNF to TrkB, as found with others evaluating BDNF and NT-3 binding to NIH 3T3 cells transfected with TrkB or TrkC (34, 48). Second, signaling through TrkB induced greater association and activation of PLC-γ1 than through TrkC. Third, TrkC signaling induced greater association and activation of SHC than TrkB signaling. How these differences arise is uncertain. The simplest possibility is that there are differences between TrkB and TrkC in recruitment and activation of signaling intermediates. Alternatively, the time courses of association of these intermediates may differ following TrkB and TrkC activation. Another possibility is that the difference is due to PLC-γ1 binding somewhat more tightly to activated TrkB than TrkC, while SHC binds with higher affinity to activated TrkC than TrkB. Tighter binding would enhance the ability to recover Trk-containing complexes following immunoprecipitation, but it would not easily explain the different amounts of activated intermediates that were detected. Differences in recruitment of PLC-γ1 and SHC would be expected to create important downstream differences in signaling. Of note, we found no apparent difference in tyrosine phosphorylation of ERK1, a step that has been placed downstream of both PLC-γ1 and SHC signaling pathways (27). Finally, the quantitative differences between BDNF and NT-3 must be interpreted cautiously since they were found in different cell lines. Although the transfected cells were originally from a single parental NIH 3T3 line and would thus provide a similar cellular context for signaling, Trk transfection could have induced differences that impact neurotrophin signaling.

An interesting finding was that ERK1 phosphorylation seemed to be maximal at BDNF and NT-4 concentrations of 25 ng/ml, much lower than the concentrations (200 ng/ml) needed to reach maximum TrkB, PLC-γ1, and SHC tyrosine phosphorylation. It was remarkable that the increased signaling seen at higher concentrations of BDNF and NT-4 was not registered in increased ERK1 phosphorylation. Whether this represents a markedly more sensitive response to TrkB

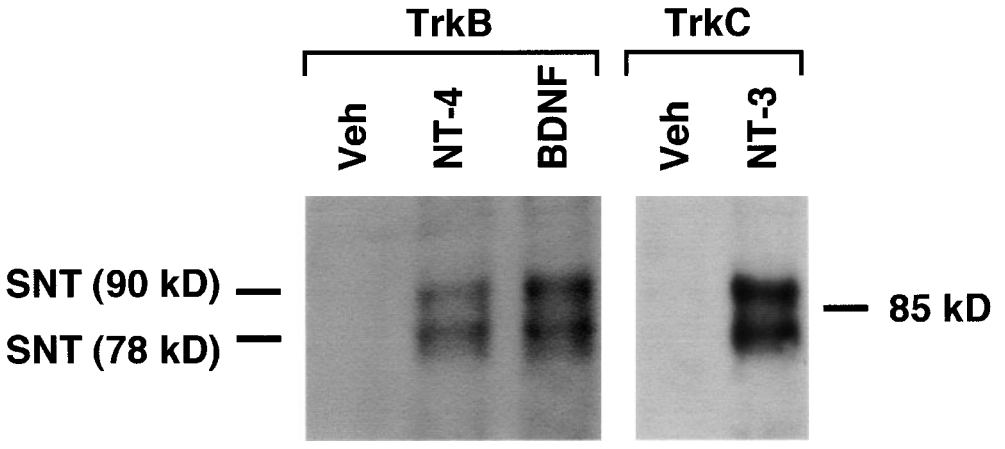


FIG. 6. SNT was tyrosine phosphorylated in TrkB- and TrkC-bearing cells in response to 5 min of treatment with BDNF (200 ng/ml), NT-4 (200 ng/ml), or NT-3 (50 ng/ml). Equal protein concentrations and volumes of cell lysates were affinity precipitated with $p13^{suc1}$-agarose. Blots were probed with anti-phosphotyrosine antibody.

activation at lower ligand concentrations or a limitation to further responsiveness with greater TrkB activation is uncertain. The former raises the possibility of signal amplification while the latter would imply the activation of mechanisms to limit ERK1 phosphorylation, such as the activation of ERK1 phosphatases (7, 43). Signal amplification would not be surprising given that TrkB activation of ERK1 occurs through at least two separate signaling pathways involving PLC-γ1 and SHC (27). These data suggest the possibility of signal amplification downstream from Trk receptors.

We were able to compare BDNF and NT-4 signaling through a single cell line that expresses TrkB. We found that BDNF and NT-4 signaling was virtually identical for TrkB phosphorylation, for the time course for decreasing TrkB phosphorylation, for the phosphorylation of PLC-γ1 and SHC and their association with TrkB, and for phosphorylation of ERK1. Although BDNF signaling and NT-4 signaling through SNT were qualitatively similar, further studies are needed to fully evaluate whether this signaling intermediate responds identically to BDNF and NT-4.

In vitro and *in vivo* studies comparing BDNF and NT-4 effects have shown both similarities (1, 6, 9, 10, 13, 15–17, 20, 21, 23, 28, 32, 33, 35, 52, 54, 55, 58, 59) and differences (2, 11, 12, 22, 29, 37, 49, 50, 53, 56). Importantly, many of the studies involving motor neurons showed similar survival effects for BDNF and NT-4 (15, 20, 21, 32, 52, 56). However, in a study of ChAT activity induced by neurotrophic factors in embryonic rat motor neuron cultures, maximal stimulation of ChAT activity by BDNF occurred at 10 ng/ml, com-

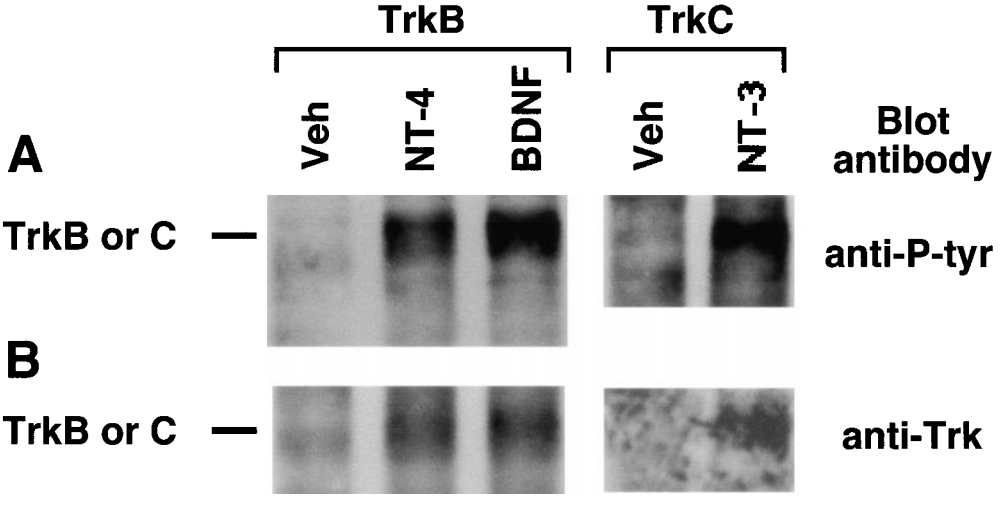


FIG. 7. PI3-kinase was associated with tyrosine phosphorylated TrkB or TrkC in response to 5 min of treatment with BDNF (200 ng/ml), NT-4 (200 ng/ml) or NT-3 (50 ng/ml). Equal protein concentrations and volumes of cell lysates were immunoprecipitated with anti-PI3-kinase (p85) antibody. Blots were probed with anti-phosphotyrosine antibody (A) and then with anti-Trk antibody (B).

pared to 100 ng/ml for NT-4 (56). In neonatal mice with sciatic nerve axotomy, BDNF promoted survival of all motor neurons, whereas NT-4 promoted survival of only a subset (37).

There are several possible explanations for the differences between BDNF and NT-4 actions in earlier studies. Many of the differences were seen in cells known to express $p75^{NTR}$, including neonatal motor, sensory, and striatal neurons. $p75^{NTR}$ may play a role in modulating TrkB signaling in different ways for BDNF and NT-4 (45). Alternatively, BDNF and NT-4 may signal differently through $p75^{NTR}$ alone. The cell line that we studied (NIH 3T3 transfected with TrkB) does not express $p75^{NTR}$. Another possibility is that different isoforms of TrkB respond differently to BDNF and NT-4. A naturally occurring splice variant of TrkB, which skips exon 9, has been shown to have increased TrkB phosphorylation and $p21^{ras}$ in response to BDNF compared to NT-4 (49). A final possibility is that subtle differences in early BDNF and NT-4 signaling that are difficult to document in studies like ours nevertheless result in important differences in cellular responsiveness far downstream. Careful additional studies of BDNF and NT-4 signaling in cells that do show different responses to these neurotrophins will be important.

In conclusion, we found qualitatively similar signaling pathways for BDNF and NT-4 through TrkB and NT-3 through TrkC in NIH 3T3 cells. Our findings showed that signaling in these cells is quite similar to what has been seen for NGF signaling through TrkA in PC12 and GT1-1 cells (60). They indicate that the Trk family of neurotrophin receptors uses a common set of early signaling intermediates over very similar time courses. This study suggests that additional experiments in well-characterized cell lines could be used to explore quantitative differences in the signal transduction cascades initiated by Trk receptor activation.

ACKNOWLEDGMENTS

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