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# Expression and Function of *TRK-B* and *BDNF* in Human Neuroblastomas

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**There is considerable interest in the role of the *TRK* family of neurotrophin receptors in regulating growth and differentiation in normal and neoplastic nerve cells. A neuroblastoma is a common pediatric tumor derived from the neural crest, and the majority of favorable neuroblastomas express a high level of *TRK-A* mRNA. However, little is known about the expression or function of *TRK-B* in these tumors. *TRK-B* encodes a tyrosine kinase that binds to brain-derived neurotrophic factor (BDNF), as well as neurotrophin-3 (NT-3) and NT-4/5. We have studied the N-*myc*-amplified human neuroblastoma cell line, SMS-KCN, which expresses both *TRK-B* and *BDNF*. Exogenous BDNF induces tyrosine phosphorylation of TRK-B as well as phosphorylation of phospholipase C-γ1, the extracellular signal-regulated kinases 1 and 2, and phosphatidylinositol-3 kinase. BDNF also induces expression of the immediate-early genes c-*FOS* and *NGFI-A* but not *NGFI-B* or *NGFI-C*. In addition, BDNF appears to promote cell survival and neurite outgrowth. SMS-KCN cells also express TRK-A, which is phosphorylated in response to nerve growth factor. However, the downstream TRK-A signaling is apparently defective. Finally, we determined that in a series of 74 primary neuroblastomas, 36% express *TRK-B* mRNA, 68% express *BDNF* mRNA, and 31% express both. Truncated *TRK-B* appears to be preferentially expressed in more-differentiated tumors (ganglioneuromas and ganglioneuroblastomas), whereas full-length *TRK-B* is expressed almost exclusively in immature neuroblastomas with N-*myc* amplification. Our findings suggest that in *TRK-B*-expressing human neuroblastomas, BDNF promotes survival and induces neurite outgrowth in an autocrine or paracrine manner. The BDNF/TRK-B pathway may be particularly important for growth and differentiation of neuroblastomas with N-*myc* amplification.**

Developing neurons require trophic factors for survival, growth, and differentiation (4, 20). Nerve growth factor (NGF) was first discovered as a neurotrophic factor that supports the development and maintenance of peripheral sympathetic and neural crest-derived sensory neurons (35, 63). NGF is a member of a family of proteins that includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) (14, 23, 25, 38, 56), and NT-4/5 (7, 19). Despite the high sequence homology, the developmental and physiological functions of each factor appear to be different.

Recently, three receptors for the neurotrophic factors of the NGF family have been cloned, and they also form a gene family encoding protein tyrosine kinases (28, 32, 33, 41–43). *TRK-A* encodes a receptor for NGF but also binds NT-3 and NT-4/5 (7, 22, 26, 29, 31, 60). *TRK-B* encodes a receptor for BDNF but also binds NT-3 and NT-4/5 (7, 31, 60), and *TRK-C* encodes a receptor for NT-3 (33). The pattern of expression of each gene or protein in the developing nervous system of the mouse is specific, suggesting that each has a unique role (9, 30, 40, 58).

Recently, we found that most favorable neuroblastomas express high levels of *TRK-A* transcripts and that neuroblastoma cells expressing *TRK-A* can differentiate in response to NGF in culture (47, 48). In contrast, aggressive neuroblastomas, especially those with N-*myc* amplification, express little or no detectable *TRK-A* mRNA, and many cell lines have a defective NGF receptor signaling pathway (2, 3, 39, 59). These results suggest that the biology of neuroblastomas is closely correlated with the developmental stages of the neurons from which the tumors originate, so neuroblastomas may be regulated in part by neurotrophic factors.

Here we report that a human neuroblastoma cell line, SMS-KCN, expresses both *TRK-B* and *BDNF* transcripts as well as *TRK-A* mRNA. BDNF induces immediate-early genes and phosphorylation of phospholipase C-γ1 (PLC-γ1), the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), and phosphatidylinositol-3 kinase (PI-3K) and appears to stimulate cell survival and neurite extension in an autocrine or paracrine manner. In contrast, NGF induces autophosphorylation of TRK-A, but the signaling pathway is deficient. Finally, truncated *TRK-B* appears to be preferentially expressed in more-differentiated tumors (ganglioneuromas and ganglioneuroblastomas), whereas full-length *TRK-B* is expressed in immature neuroblastomas with N-*myc* amplification.

## MATERIALS AND METHODS

**Cells and tissues.** The derivations, descriptions, and culture conditions of cell lines were described previously (2). SMS-KCN cells, a gift from C. Patrick Reynolds (55), were maintained in the RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin (standard medium) at 5% $CO_2$–95% air at 37°C. Fresh tumor samples were obtained from 59 Japanese patients and 15 patients of the Pediatric Oncology Group in the United States. These 74 tumors were frozen on dry ice or in

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liquid nitrogen immediately after surgery (47, 48) and were stored at −80°C until use. Staging was done according to the method of Evans and colleagues (17). Excluding the 5 ganglioneuromas, 18 of the tumors were stage I, 11 were stage II, 12 were stage IV-S, 14 were stage III, and 14 were stage IV. Histologically, 5 were ganglioneuromas, 16 were ganglioneuroblastomas, and 53 were neuroblastomas. The histologies of the tumors were classified according to the guidelines proposed by the Japanese Pathological Society (54), which were based solely on the histological grade of differentiation of the neuroblastomas without involving age or other factors.

**Neurotrophins, cDNA probes, and drugs.** The following probes and reagents were generous gifts: recombinant human BDNF and NT-3 from AMGEN Pharmaceutical Co., Ltd.; mouse NGF and anti-NGF antibody from Eugene M. Johnson; the human *BDNF* probe from George D. Yancopoulos; the N-*myc* probe from J. Michael Bishop; probes for *TRK-A* and *TRK-B* from Luis Parada; the low-affinity nerve growth factor receptor (LNGFR) full-length cDNA probe from Moses Chao; and probes for human *NGFI-A*, *NGFI-B*, and *NGFI-C* from Jeffrey D. Milbrandt. The *TRK-A* probe was a 2.7-kb human cDNA fragment containing all of the coding region. The mouse *TRK-B* probe pFRK16 (32) recognizes mRNA transcripts both with and without the kinase domain (28, 43). The rat *TRK-C* probe pJDM836 contains a 557-bp PCR fragment corresponding to nucleotides 272 to 829 of the porcine *TRK-C* cDNA (33). K252a, an inhibitor of tyrosine phosphorylation (6, 52, 62), was purchased from Calbiochem Corp., La Jolla, Calif.

**Northern (RNA) blot analysis.** Total RNA was extracted from 0.2 to 1.0 g of cultured cells or frozen tumor tissue as described previously (10). We resolved 25 μg of each RNA on 1% agarose-formaldehyde gels and transferred the RNA by blotting to a nylon membrane (Hybond N+; Amersham, Arlington Heights, Ill.). Blots were hybridized, washed, and exposed to X-ray film as described previously (57). Northern analysis for expression of *TRK-B* and *BDNF* in the primary neuroblastomas was repeated three times.

**Immunoprecipitations and immunoblots.** Cells were grown in 15-cm-diameter dishes to ~90% confluency and incubated in serum-free medium for 1 h prior to NGF treatment. NGF-treated (100 ng/ml for 5 min) or untreated cells were then washed once with ice-cold Tris-buffered saline (TBS) (10 mM Tris [pH 8.0], 150 mM NaCl) and harvested by the addition of 300 μl of ice-cold lysis buffer (1% Nonidet P-40, 1 mM $NaVO_4$, 0.1 mM $NaMoO_4$, 1 mM phenylmethylsulfonyl fluoride, 3.3 μM pepstatin, 2 μM Bestatin, 10 μM leupeptin, 5.25 μg of aprotinin per ml, 0.02% $NaN_3$ in TBS) directly to the cell monolayer. BDNF- or NT-3-treated cells were exposed to 100 ng of BDNF or NT-3 per ml and treated in a manner similar to that for NGF-treated cells. Lysates were then immediately frozen and stored at −80°C. For the K252a studies, cells were incubated in serum-free medium with or without different concentrations of K252a for 6 h before the addition of 100 ng of BDNF per ml.

Prior to immunoprecipitation, lysates were thawed and incubated at 4°C for 15 min to ensure effective lysis, and cell debris was pelleted in a microcentrifuge (Eppendorf). Lysate supernatants were precleared by 1-h incubations with preimmune rabbit serum and immobilized protein A bound to Sepharose beads (Repligen), in that order. The beads were spun down; anti-TRK-A antiserum (a generous gift from Luis Parada) or anti-TRK-A polyclonal immunoglobulin G (Santa Cruz Biotechnology, Inc.), both of which recognize both $p140^{TRK-A}$ and $p145^{TRK-B}$, was added to the supernatants; and incubations were performed at 4°C for ~16 h. Protein A beads were then added, and after 1 h at 4°C, the beads were pelleted and washed three times with lysis buffer and three times with TBS. The beads were then resuspended in 1× Laemmli sample buffer and boiled for 5 min. The immunoprecipitates were then loaded onto 7.5% polyacrylamide gels for electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon) in cold 192 mM glycine–25 mM Tris base–20% (vol/vol) methanol for 45 min at 1 A. The blots were treated with 2% gelatin in TBS for ~45 min and were washed and then incubated with 1 μg of antiphosphotyrosine monoclonal antibody (UBI, Lake Placid, N.Y.) per ml in TBS–0.05% Tween 20 overnight. The antibody specifically bound to phosphotyrosine residues was visualized with an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G secondary antibody (BMG) and a Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium detection system (Promega). These immunoprecipitates were then blotted, and the blot was probed with antiphosphotyrosine antibody (UBI).

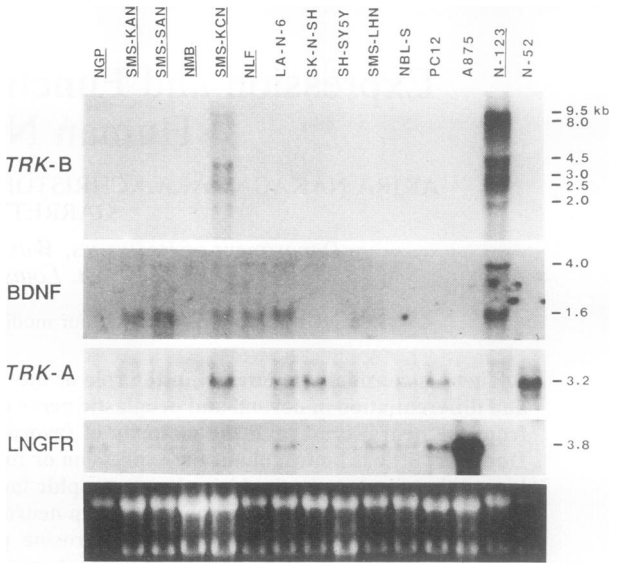


FIG. 1. Expression of *TRK-B*, *BDNF*, *TRK-A*, and *LNGFR* in 11 neuroblastoma cell lines, the PC12 rat pheochromocytoma cell line, and the A875 human melanoma cell line. For comparison of levels of *TRK-B* expression in primary tumors, total RNAs from a stage III primary neuroblastoma with N-*myc* amplification (N-123) and a stage I primary neuroblastoma (N-52) were loaded in the same gel. The underlines show the neuroblastoma cell lines or the primary tumor with N-*myc* amplification. The bottom panel is a photograph of the ethidium bromide-stained Northern blot gel.

## RESULTS

**Expression of neurotrophins and their receptors in neuroblastoma cell lines.** Expression of *TRK-A*, *TRK-B*, *TRK-C*, *LNGFR*, *NGF*, and *BDNF* mRNAs in 11 human neuroblastoma cell lines, the PC12 rat pheochromocytoma cell line, and the A875 human melanoma cell line was studied by Northern blot analysis (Fig. 1). We also studied neurotrophic factor and receptor expression in a series of 74 neuroblastomas (see below). None of the cell lines except SMS-KCN (with N-*myc* amplification) expressed readily detectable levels of *TRK-B* transcripts. At least six different sizes of

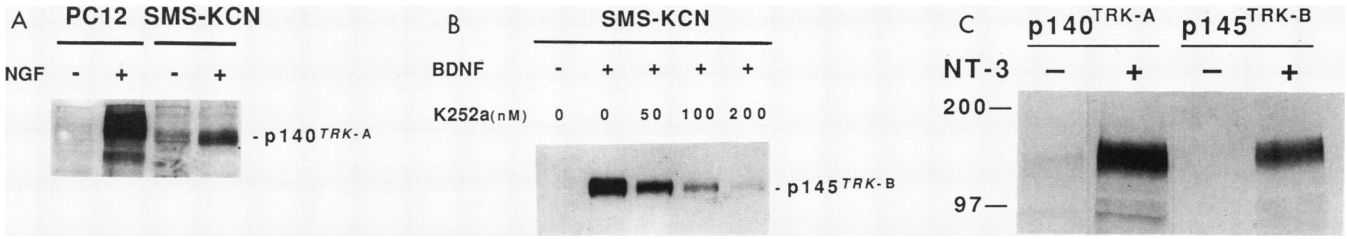


FIG. 2. Tyrosine phosphorylation of p140$^{TRK-A}$ and p145$^{TRK-B}$ in SMS-KCN human neuroblastoma cells. (A) Cells were either untreated (−) or treated with 100 ng of NGF per ml for 5 min at 37°C (+). The cell lysates were immunoprecipitated with anti-TRK-A antibody and then blotted with antiphosphotyrosine antibody (see Materials and Methods). (B) SMS-KCN cells were treated with 100 ng of BDNF per ml for 5 min at 37°C after incubation with the indicated concentrations of K252a for 6 h. The cell lysates were immunoprecipitated with anti-TRK-B antibody and blotted with antiphosphotyrosine antibody. (C) Cells were treated with 100 ng of NT-3 per ml for 5 min at 37°C. The cells were then immunoprecipitated with anti-TRK-A antibody or anti-TRK-B antibody and then blotted with antiphosphotyrosine antibody.

human *TRK-B* transcripts were detected in this cell line: 9.5, 8.0, 4.5, 3.0, 2.5, and 2.0 kb. BDNF, the primary ligand of p145$^{TRK-B}$, was expressed in four N-*myc*-amplified neuroblastomas (SMS-KAN, SMS-SAN, SMS-KCN, and NLF) and two nonamplified neuroblastomas (LA-N-6 and SMS-LHN). Two other neuroblastomas with N-*myc* amplification (NGP and NMB) also showed weak expression of *BDNF*. Neither *TRK-B* expression nor *BDNF* expression was detected in the SK-N-SH, SH-SY5Y, NBL-S, PC12, or A875 cell line.

Surprisingly, SMS-KCN cells expressed *TRK-A* at a level similar to that of PC12 cells, although *TRK-A* expression generally is down-regulated in N-*myc*-amplified neuroblastomas (47). NGF expression was observed only in the primary neuroblastoma N-123 (see below) but not in any cell lines tested (data not shown). LNGFR was expressed in all cell lines except five N-*myc*-amplified neuroblastoma cell lines (SMS-KAN, SMS-KCN, SMS-SAN, NMB, and NLF). None of the cell lines or primary neuroblastomas tested showed a detectable level of *TRK-C* expression, as measured by Northern analysis (data not shown).

**BDNF induces phosphorylation of p145$^{TRK-B}$ and expression of immediate-early genes in SMS-KCN cells.** With SMS-KCN cells, NGF induced phosphorylation of p140$^{TRK-A}$ (Fig. 2A) and BDNF induced phosphorylation of p145$^{TRK-B}$ (Fig. 2B). The phosphorylation of p145$^{TRK-B}$ induced by BDNF was inhibited by K252a in a dose-dependent manner, and inhibition was almost complete at 200 nM (Fig. 2B), which is similar to reports of inhibition of NGF-induced phosphorylation of p140$^{TRK-A}$ (6, 62). The addition of NT-3 to SMS-KCN cells also induced receptor phosphorylation recognized by both anti-p140$^{TRK-A}$ and anti-p145$^{TRK-B}$ antibodies (Fig. 2C; see below). NGF induced none of the immediate-early genes in SMS-KCN cells (Fig. 3A), whereas BDNF, as well as NT-3, induced c-*FOS* and *NGFI-A* but not *NGFI-B* or *NGFI-C* (Fig. 3A and B).

These results contrast with the effect of NGF on PC12 cells, which is presumably mediated by p140$^{TRK-A}$. NGF induces phosphorylation of p140$^{TRK-A}$ (26, 27, 29) and expression of immediate-early genes such as c-*FOS* (13, 18, 44), *NGFI-A/egr1/zif/268* (11, 45, 61), *NGFI-B/nur77* (21, 46), and *NGFI-C* (12). Primary cultures of neuroblastoma cells which express both functional p140$^{TRK-A}$ and p75$^{LNGFR}$ show a response to NGF stimulation similar to that of PC12 cells (48). However, no induction of immediate-early genes was seen in SMS-KCN cells (or other neuroblastoma cell lines tested) in response to NGF. Furthermore, BDNF and NT-3 induced c-*FOS* and *NGFI-A* but not *NGFI-B* or *NGFI-C*.

To perform these studies, we used two anti-p140$^{TRK-A}$ antibodies from different sources, both of which recognized both p140$^{TRK-A}$ and p145$^{TRK-B}$ (data not shown). In contrast, the anti-p145$^{TRK-B}$ antibody obtained from Santa Cruz Bio-

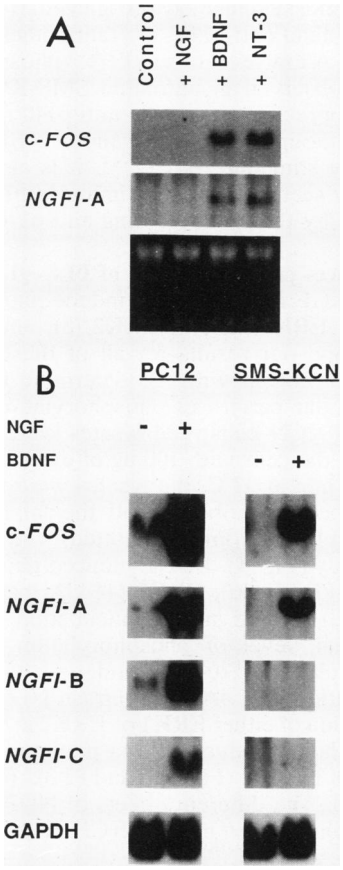


FIG. 3. Induction of expression of immediate-early genes in SMS-KCN cells. (A) Induction of c-*FOS* and *NGFI-A* in SMS-KCN cells in response to NGF, BDNF, or NT-3. Cells were treated with 100 ng of NGF, BDNF, or NT-3 per ml for 40 min at 37°C. The bottom panel is a photograph of the ethidium bromide-stained gel used for the Northern blotting. (B) Effect of NGF (in PC12 cells) or BDNF (in SMS-KCN cells) on induction of immediate-early genes c-*FOS*, *NGFI-A*, *NGFI-B*, and *NGFI-C*. Cells were treated with 100 ng of NGF or BDNF per ml for 40 min. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression is shown for the normalization of expression of the other genes.

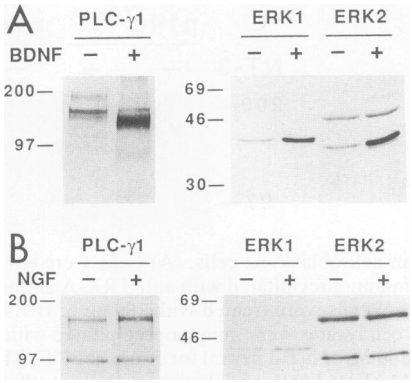


FIG. 4. Tyrosine phosphorylation of PLC-γ1, ERK1, and ERK2 before (−) and after (+) the addition of BDNF (100 ng/ml) (A) or NGF (100 ng/ml) (B) to SMS-KCN cells for 5 min. Lysates were immunoprecipitated with specific antibody to each protein and blotted with antiphosphotyrosine antibody (see Materials and Methods).

technology almost exclusively recognized p145$^{TRK-B}$ but was less sensitive than anti-p140$^{TRK-A}$ antibodies in recognizing this receptor. A low level of p140$^{TRK-A}$ phosphorylation was consistently observed in untreated SMS-KCN cell lysates after immunoprecipitation with anti-p140$^{TRK-A}$ antibodies (Fig. 2A). Although some endogenous NGF- or NT-3-like molecule may stimulate p140$^{TRK-A}$, it is also possible that endogenously secreted BDNF stimulates p145$^{TRK-B}$, which is recognized by the cross-reacting anti-p140$^{TRK-A}$ antibodies.

**BDNF induces phosphorylation of PLC-γ1, ERK1, ERK2, and PI-3K.** NGF is reported to induce phosphorylation of PLC-γ1 (64), ERK1 (8, 37), ERK2 (8), and PI-3K (53) in PC12 cells, and our results for all of these proteins were identical (data not shown). To determine whether BDNF induces a similar pattern of phosphorylation of these proteins in SMS-KCN cells, cell lysates were prepared after incubation of the cells with 100 ng of exogenous BDNF per ml for 1 or 5 min at 37°C, the lysates were immunoprecipitated with specific antibody, and the immunoprecipitates were Western blotted (immunoblotted) with antiphosphotyrosine antibody (Fig. 4). BDNF induced phosphorylation of PLC-γ1 (150-kDa band), ERK1 (44-kDa band), and ERK2 (42-kDa band) after 5 min of incubation and induced a relatively weak level of phosphorylation of PI-3K (one 85-kDa band plus one 110-kDa band; data not shown) after 1 min of incubation (Fig. 4A). In contrast, NGF did not induce phosphorylation of either ERK1 or ERK2 in SMS-KCN cells (Fig. 4B), although induction of a minimum level of phosphorylation of PLC-γ1 (Fig. 4B) and PI-3K (data not shown) was observed. The different effects of NGF and BDNF on protein phosphorylation in SMS-KCN cells are summarized in Table 1.

**Effect of exogenous BDNF on growth and differentiation of SMS-KCN cells in vitro.** The effect of exogenous neurotrophic factors (100 ng/ml) on the growth of SMS-KCN cells is shown in Fig. 5A. BDNF slightly increased the cell number during culture for longer than 7 days, while neither NGF nor NT-3 had a significant effect as determined by comparison with control cultures. Cell cycle analysis by flow cytometry showed that the treatment of SMS-KCN cells with 100 ng of exogenous BDNF per ml had no appreciable effect on the percentage of cells in different phases of the cell cycle (data not shown). This suggests that the increase in the cell number may be due to promotion of survival rather than stimulation of cell division.

TABLE 1. Summary of protein phosphorylation in the BDNF/TRK-B signal transduction pathway in the SMS-KCN neuroblastoma cell line

| Protein phosphorylated | Phosphorylation[a] by: | | | |
|---|---|---|---|---|
| | SMS-KCN | | PC12 | |
| | NGF | BDNF | NGF | BDNF |
| TRK-A | + | (−) | + | − |
| TRK-B | − | + | − | − |
| PLC-γ1 | ± | + | + | ND |
| ERK1 | − | + | + | ND |
| ERK2 | − | + | + | ND |
| PI-3K | ± | ± | + | ND |

[a] +, strong induction; ±, weak induction; −, no induction; (−), negative result which cannot be confirmed absolutely because of cross-reactivity of TRK-A antibody with p145$^{TRK-B}$; ND, not determined.

We addressed the potential influence of serum concentration on cell growth or differentiation. SMS-KCN cells were grown at decreased serum concentrations in the culture medium, and as shown in Fig. 5B, cell growth was inhibited in a dose-dependent manner. However, the addition of exogenous BDNF increased the cell number at all serum concentrations. Even in the serum-free medium, there was half the starting number of cells after 8 days of culture with exogenous BDNF, though most cells were dead in its absence.

At every serum concentration, the presence of exogenous BDNF appeared to stimulate neurite outgrowth. In control medium with 10% serum, SMS-KCN cells spontaneously extended short neurites (Fig. 6A), which became straighter and longer as the cell density became higher. There appeared to be modest enhancement of neurite outgrowth in the presence of exogenous BDNF (Fig. 6B), but it was difficult to document. In medium with 0.5% serum, however, the cells survived without an increase in the cell number, and spontaneous neurite outgrowth was very limited (Fig. 6C). The addition of exogenous BDNF clearly increased the cell number and markedly enhanced neurite outgrowth (Fig. 6D).

**Expression pattern of *TRK-B* and *BDNF* mRNAs in primary neuroblastomas.** In contrast to results with neuroblastoma cell lines, expression of *TRK-B* was observed in 27 of 74 (36%) primary neuroblastomas (including five ganglioneuromas), but the expression pattern was complex (Fig. 7). By extrapolation from studies of the mouse and rat *TRK-B* mRNA species (32, 43), the ~9.5- and ~4.5-kb transcripts presumably encode the full-length product of human *TRK-B*, and other size transcripts encode the truncated form of the receptor without the tyrosine kinase domain. In each primary neuroblastoma expressing *TRK-B*, the intensities of both the ~9.5- and ~4.5-kb transcripts appeared to be equivalent. A clear ~9.5-kb transcript was found in 7 of 10 (70%) neuroblastomas with N-*myc* amplification and in only 2 of 64 (3%) tumors without amplification ($\chi^2 = 30.22$; $P < 0.001$) (Table 2). On the other hand, preferential expression of the putative truncated forms, especially of the ~8.0-kb transcript, was observed in 18 of 64 (28%) neuroblastomas without N-*myc* amplification. Interestingly, five of five (100%) ganglioneuromas showed preferential expression of the putative truncated form of *TRK-B* mRNA (Fig. 7; Table 2).

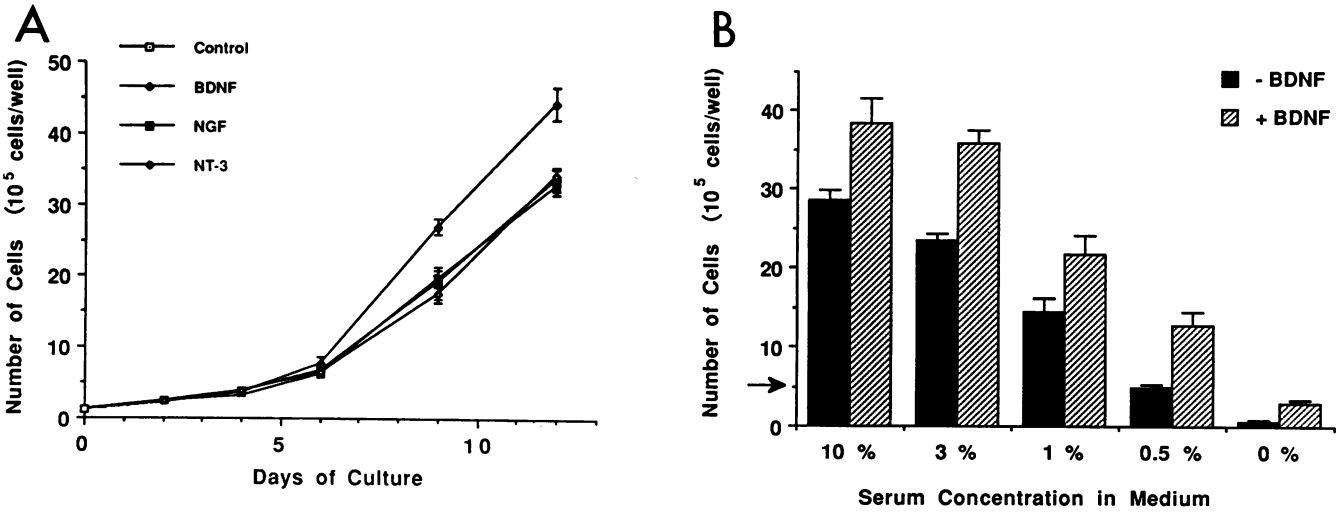


FIG. 5. (A) Effects of NGF, BDNF, and NT-3 on the growth of SMS-KCN cells. A total of $10^5$ cells were seeded in a 24-well tissue culture plate (15-mm diameter; Falcon) and maintained in the complete medium, with changing every 2 to 3 days. The final concentration of each neurotrophic factor added was 100 ng/ml. The number of adherent cells was counted on day 8. Values are shown as means ± standard deviations ($n$ = 4). (B) Effects of serum concentrations on the growth of SMS-KCN cells in the presence or absence of 100 ng of BDNF per ml. A total of $5 \times 10^5$ cells per well (35-mm-diameter plate; Falcon) were plated on day 0 (arrow). Values for cell numbers on day 8 are shown as means ± standard deviations ($n$ = 4).

*BDNF* mRNA expression was detected in 50 of 74 (68%) primary neuroblastomas (Table 2). The frequencies were somewhat higher in advanced-stage (III or IV) neuroblastomas (22 of 28; 79%) and in mature ganglioneuromas (5 of 5; 100%) than in favorable-stage (I, II, or IV-S) neuroblastomas (23 of 41; 56%). Overall, 31% of the tumors had concordant expression of both *TRK-B* and *BDNF*. Seven of ten tumors with N-*myc* amplification had expression of both putative

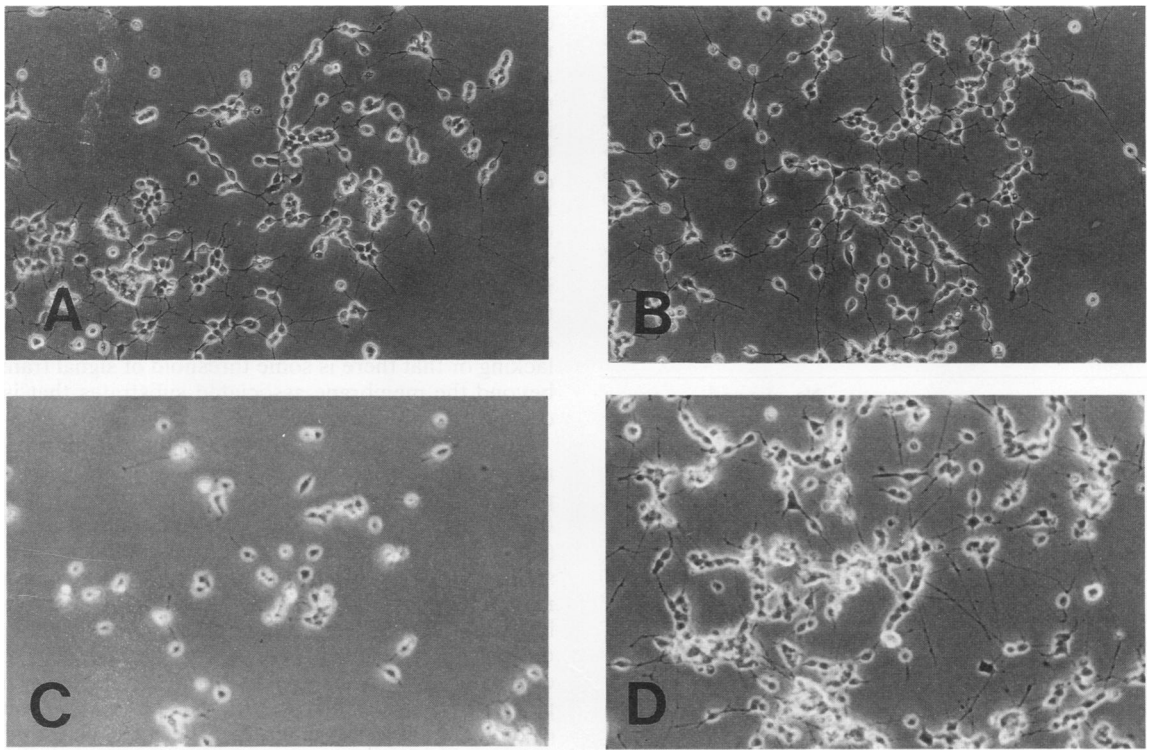


FIG. 6. Effects of BDNF on SMS-KCN cells cultured in the complete medium or in the 0.5% serum medium. (A and B) Cells were cultured in the 10% serum–RPMI 1640 medium for 5 days at 37°C at 5% $CO_2$–95% air without (A) or with (B) 100 ng of BDNF per ml. The initial cell concentration was $2 \times 10^5$ cells per ml. (C and D) Cells were cultured in the 0.5% serum medium alone (C) or with BDNF (D) for 8 days. The initial cell concentration was $5 \times 10^5$ cells per ml.

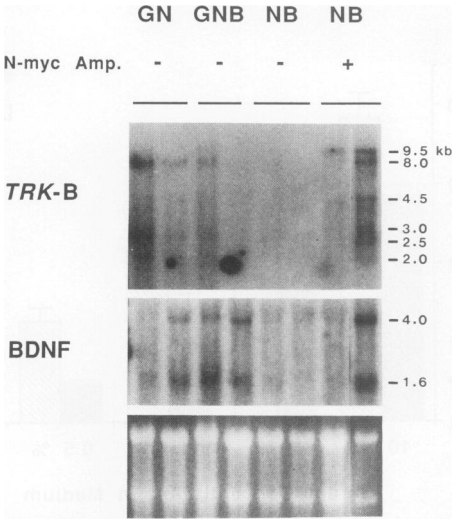


FIG. 7. Expression of *TRK-B* and *BDNF* in representative primary neuroblastomas. The bottom panel shows the ethidium bromide-stained gel used for the Northern blotting. GN, ganglioneuroma; GNB, ganglioneuroblastoma; NB, neuroblastoma; Amp., amplification.

full-length *TRK-B* and *BDNF*, similar to the expression in the SMS-KCN cell line. Of the remaining 64 tumors, 16 (25%) had coexpression of *BDNF* and *TRK-B*, and all but 2 of these expressed predominantly truncated forms.

## DISCUSSION

**The BDNF/TRK-B signaling pathway in the SMS-KCN neuroblastoma cell line is functional.** In contrast to the NGF/TRK-A pathway, the BDNF/TRK-B signaling pathway in neuronal cells has been unclear because a suitable cell line has not been available. In the present article, we report that an N-*myc*-amplified neuroblastoma cell line, SMS-KCN, expresses readily detectable endogenous levels of both *TRK-B* and *BDNF* mRNAs. This cell line also expresses *TRK-A* but not *TRK-C*, *LNGFR*, or *NGF*.

In SMS-KCN cells, exogenous BDNF induced phosphorylation of p145$^{TRK-B}$ and the downstream signaling pathway, including phosphorylation of PI-3K after 1 min of incubation, phosphorylation of PLC-γ1, ERK1, and ERK2 after 5 min of incubation, and induction of the immediate-early genes c-*FOS* and *NGFI-A*. The signaling cascade is very similar to that of the NGF/TRK-A system in PC12 rat pheochromocytoma cells (8, 37, 53, 64), except for the absence of induction of *NGFI-B* and *NGFI-C*. Since *NGFI-B* is expressed in many primary neuroblastoma tissues (unpublished observations), the failure of this gene to be induced is probably not due to cell type or species differences.

TABLE 2. Expression of TRK-B and BDNF in primary neuroblastomas

| No. of N-*myc* copies | Histology | No. of cases[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Total | With TRK-B mRNA expression | | | With BDNF mRNA expression | |
| | | | − | + (full) | + (trunc.) | − | + |
| 1 | Ganglioneuroma | 5 | 0 | 0 | 5 | 0 | 5 |
| | Ganglioneuroblastoma | 15 | 10 | 0 | 5 | 6 | 9 |
| | Neuroblastoma | 44 | 34 | 2 | 8 | 17 | 27 |
| >1 | Neuroblastoma | 10 | 3 | 7 | 0 | 1 | 9 |
| Total | | 74 | 47 | 9 | 18 | 24 | 50 |

[a] −, absent; +, present; full, tumors had ~9.5-kb signal intensity greater than or equal to the ~8.0-kb signal intensity; trunc., tumors had ~9.5-kb signal intensity less than the ~8.0-kb signal intensity.

BDNF seemed to promote cell survival and to induce neurite outgrowth of SMS-KCN cells. However, there are striking differences between the BDNF/TRK-B system in SMS-KCN cells and the NGF/TRK-A system in PC12 cells. BDNF may act on the SMS-KCN cells in an autocrine or paracrine manner, though it may not be saturated, while PC12 cells require exogenous NGF in order to differentiate. In addition, BDNF seems to induce neurite outgrowth and survival in SMS-KCN cells without substantial effects on cell growth, while NGF causes a decrease in the growth rate of PC12 cells. The presence of a BDNF/TRK-B autocrine loop in central and peripheral nervous systems has been suggested recently by some investigators on the basis of in situ hybridization studies (15, 58). Most primary neuroblastomas expressing both *BDNF* and *TRK-B* have N-*myc* amplification and overexpression, as shown in Table 2, and this is true for the SMS-KCN cell line as well. Thus, the potential autocrine stimulation of these cells through the TRK-B/BDNF pathway may be a consequence of N-*myc* overexpression.

SMS-KCN cells express TRK-A receptors as well, and NGF-induced phosphorylation of p140$^{TRK-A}$ occurs. However, the downstream signaling cascade, including phosphorylation of ERK1 and ERK2 as well as induction of c-*FOS* and *NGFI-A*, was deficient. NGF also had no effect on morphological differentiation or tumor cell growth. Thus, as in some other neuroblastoma cell lines (2, 3, 39, 59), the NGF/TRK-A signal transduction pathway in SMS-KCN cells seems to be defective, although the similar signaling BDNF/TRK-B pathway is intact. PLC-γ1 and PI-3K were weakly phosphorylated by the treatment of SMS-KCN cells with NGF. One of the explanations for this may be that there is a close association of these molecules with p140$^{TRK-A}$ near the cell membrane (53). It is also possible that some signaling intermediate required for immediate-early gene induction is lacking or that there is some threshold of signal transduction beyond the membrane-associated substrates that is not exceeded by NGF interacting with its receptor.

The exogenously added NT-3 induced expression of immediate-early genes in SMS-KCN cells, probably through phosphorylation of the p145$^{TRK-B}$ receptor (Fig. 2C). Although NT-3 also induced phosphorylation of p140$^{TRK-A}$ (Fig. 2C), NGF was unable to induce immediate-early genes, so it is less likely that NT-3 would do so through this receptor. It is also possible that TRK-C was expressed below the level of detection or that another unknown receptor capable of binding NT-3 was responsible. However, the evidence that NT-3 could induce phosphorylation of p145$^{TRK-B}$ and the fact that BDNF can induce immediate-early gene induction make this the most plausible mechanism.

**Neuroblastomas expressing *TRK-A*, *TRK-B*, and/or *BDNF* may represent stages of the normal developmental neuronal lineage.** The discovery of genes encoding neurotrophic fac-

tors and their receptors has provided considerable insight into the biology of neuroblastomas. In a previous study, we found that most primary neuroblastomas with favorable prognoses expressed a very high level of *TRK-A* mRNA, usually together with *LNGFR* mRNA. These tumor cells responded to NGF by terminally differentiating in vitro and died in the absence of NGF (47, 48). These observations suggested that tumor cells expressing functional NGF receptor may be susceptible to either programmed cell death, resulting in tumor regression, or to differentiation, leading to a benign ganglioneuroma, in vivo.

Our present results further show that many aggressive neuroblastomas, especially those with N-*myc* amplification, expressed both putative full-length *TRK-B* and *BDNF* mRNAs, although generally the expression of *TRK-A* mRNA was extremely low or absent. The almost mutually exclusive expression of *TRK-A* and *TRK-B* (with or without *BDNF*) suggests that neuroblastomas may be categorized into distinct subsets. Favorable tumors are composed mainly of cells expressing *TRK-A*, while aggressive tumors are composed of cells expressing both *TRK-B* and *BDNF*, often accompanied by N-*myc* amplification.

This hypothesis concerning the differential expression of *TRK-A* and *TRK-B* in neuroblastomas is consistent with the observations that neural crest-derived dorsal root ganglion cells normally require NGF and BDNF, as well as NT-3 and NT-4/5, to survive (5, 19, 23, 36). Furthermore, *TRK-A* and *TRK-B* transcripts are observed in distinct subsets of neurons in the dorsal root ganglia and sympathetic ganglia, as seen by in situ hybridization (9, 30, 40, 58). Finally, *BDNF* and/or *NT-3* transcripts are present in many neurons in both dorsal root ganglia and sympathetic ganglia, whereas *NGF* transcripts are not (16, 58). Thus, BDNF may stimulate responsive neurons by a local mechanism (15, 58), and it may be particularly important in providing trophic support to sensory neurons during the earliest phases of target innervation.

In the normal neuronal lineage, BDNF appears to support the survival of dopaminergic neurons (24). It is interesting that aggressive neuroblastomas with N-*myc* amplification often have dopaminergic characteristics (34, 49–51) and that those tumors often express both *TRK-B* and *BDNF*. In contrast, the putative truncated form of human *TRK-B* is preferentially expressed in more-differentiated ganglioneuroblastomas and ganglioneuromas. It is not clear whether the *TRK-B* expression of such tumors is derived from the differentiated neuroblastic tumor cells or from the Schwannian elements that are present in the differentiated tumors, because Schwann cells also are reported to express *TRK-B* and *BDNF* (1, 9). The roles of *NT-3* and *TRK-C* in neuroblastomas remain unclear. Remarkably, some primary cultures of neuroblastoma cells responded to NT-3 in a manner similar to that for NGF (unpublished observations). However, we have not detected expression of *TRK-C* in any primary neuroblastomas or cell lines (data not shown). This suggests that NT-3 may be acting through *TRK-A*, *TRK-B*, or another receptor.

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