Brain-derived neurotrophic factor (BDNF) promotes neuronal survival and protection against neuronal damage. We addressed whether BDNF might promote survival and chemoprotection in neuroblastoma (NB) using a drug-sensitive human NB cell line. All-trans-retinoic acid (ATRA) induces a striking phenotypic differentiation of NB1643 cells, and exogenous BDNF treatment promotes survival of these differentiated cells. ATRA induces TRKB expression, and exogenous BDNF stimulates both autophosphorylation of TRKB and induction of the immediate early gene, FOS, in these cells. BDNF mRNA is expressed in NB1643 cells. Because the time course of TRKB induction closely parallels phenotypic differentiation of these cells, it seems probable that ATRA induces differentiation of NB1643 cells by establishing an autocrine loop involving BDNF and TRKB. Exogenous BDNF treatment resulted in a further increase in neurite outgrowth, which again suggests that an autocrine loop is involved in differentiation of NB1643 cells in response to ATRA. We then tested whether BDNF might afford drug resistance in NB and found that BDNF does indeed protect in this NB model against cisplatin, a DNA-damaging agent actually used in the treatment of NB.

Neuroblastoma (NB) is a pediatric solid tumor that arises most commonly from sympathetic precursor cells in the adrenal medulla and, to a lesser extent, from precursors of ganglion cells in the spinal cord (1). Examination of the expression of enzymes involved in chromaffin cell differentiation suggests that NB tumor cells arising from the adrenal medulla may be derived from neuroblasts arrested during a stage of morphogenesis (2). NB cells characteristically express neuronal markers, such as neuron-specific enolase (3), tyrosine hydroxylase, and dopamine β-hydroxylase (4). This suggests that NB may arise from a neuroblast that either fails to differentiate or is not eliminated by programmed cell death at the appropriate stage of development. These hypotheses encompass potentially inappropriate activation of signaling pathways that could result in failure to properly execute differentiation or cell death during development. This could lead to a proliferative disease. If a subpopulation of NB cells is refractory to chemotherapy due to activation of signaling pathways required for survival in sympathetic neurons, then an understanding these signaling pathways may prove invaluable. Chemotherapeutic strategies for preventing the arising of drug-resistant tumors or sensitizing tumors to existing drugs can be envisioned.

Neurotrophin receptors, TRK, TRKB, and, TRKC are required for development of the sympathetic nervous system (5). TRK encodes the receptor for NGF (6–8), the archetypal member of the neurotrophin family, which consists of NGF, BDNF, NT-3, and NT-4/5. The TRK family (TRKs), which consists of TRK, TRKB, and TRKC, are receptor protein-tyrosine kinases. TRKB encodes a receptor for BDNF (9–11) and NT-4/5 (12), although NT-3 also activates TRKB (9, 13). NT-3 is the ligand for TRKC receptor (14). Besides a full-length receptor protein-tyrosine kinase, TRKB expresses truncated receptors containing the extracellular region and the transmembrane domain but lacking the kinase domain (15, 16). The role that truncated receptors have is not known, although a potential role in signaling has been proposed based on the finding that BDNF activation of either TRKB.T1 or TRKB.T2 increases the rate of acidic metabolite release from cells (17).

Genetic disruption of TRK in mouse caused neuronal cell loss in sympathetic ganglia and trigeminal and dorsal root ganglia (5). Disruption of the TRKB locus in mice did not have a pronounced effect on sympathetic neurons (18), although TRKB is expressed in sympathetic neurons (19). However, TRKB is required for normal development of neurons in the peripheral nervous system (trigeminal and dorsal root ganglia) (20). In these TRKB (−/−) mice, there is an increase in neuronal loss in the trigeminal ganglion earlier than neuronal loss observed in mice homozygous for a null mutation of TRK (21). This suggests that TRKB signaling is required for survival of many trigeminal neurons before they become NGF-dependent. In the central nervous system, additional studies on TRKB (−/−) mice indicate increased apoptotic cell death in different regions of the brain, most significantly the dentate gyrus, during early postnatal life (22). TRKB may have a neuroprotective function as well. Experiments that offer direct support of this concept in vivo demonstrated lowered rates of survival for axotomized hippocampal and motor neurons in TRKB (−/−) mice contrasted to wild-type mice (22). So not only is TRKB important for survival of neurons during postnatal development, but it may well protect neurons from injury and axotomy-induced cell death.

With regard to NB, we wanted to test whether BDNF and its receptor, TRKB, have a role in survival and, perhaps more importantly, to assess whether TRKB may protect NB cells...
from chemotherapeutic drugs. Neurotrophin receptor expression could, in theory, render a population of NB cells refractory to chemotherapy and contribute to the development of drug-resistant tumors. Indeed, there are data suggesting that TRKB, and BDNF, are expressed in more aggressive tumors that have N-MYC amplification (23). In vitro data with a NB cell line indicate that BDNF may increase invasiveness of NB cells (24). In this communication, we examine the role of BDNF in promoting survival of NB cells with N-MYC amplification derived from a stage D NB tumor and present the novel finding that BDNF protects NB cells from cisplatin, a cytotoxic agent actually used in the treatment of NB.

**EXPERIMENTAL PROCEDURES**

**Reagents and Cell Culture—** 5 x 10^6 ATRA (Sigma) and 9-cis-RA (Hoffman-La Roche) stocks were prepared by dissolving retinoic acids in ethanol and stored for less than 3 months at -80°C. Human BDNF and NGF were generously supplied by AMGEN. Unless otherwise stated, NB1643 cells were grown in 10% FBS/RPMI (RPMI 1640 medium containing 2 mM glutamine (BioWhittaker) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and 50 units/ml penicillin, 50 μg/ml streptomycin) (Life Technologies, Inc.) at 37°C with 5% CO_2 in tissue culture plates from Costar. Cells were trypsinized 18 h later for use in trypan blue/acetocarmine (BDNF) for routine counting and splitting. Nuclei were prepared using the method of Butler (25) and counted using a 22 counter equipped with a 256 channelizer (Coulter) for both cell plating and for growth and survival assays monitored by nuclei counting. Poly-lysin coating of tissue culture plates was accomplished by incubating plates 16 h with 1 ml of 50 μg/ml poly-lysin in H_2O and then washing once with H_2O, air drying under UV light, and subsequently cross-linking using UV light supplied by a Stratallinker (Stratagene) using the "auto" setting.

**Differentiation of NB1643 Cells—** NB1643 cells were plated at a density of 2.5 x 10^5 per well in 6-well tissue culture dishes (Costar) with or without 5 μM ATRA in 10% FBS/RPMI medium. Final ethanol concentration was 0.1% in all wells. Photographs at a magnification of x225 were taken at 5 days following ATRA treatment. To examine the effect of exogenous BDNF treatment, NB1643 cells were plated at a density of 1 x 10^5 cells/well in 6-well dishes with 5 μM ATRA. After 3 days, medium was changed to 5 μM ATRA with or without 50 ng/ml BDNF. Final ethanol concentration was always 0.1% in all wells. Photographs at a magnification of x20 were taken 12 days after exogenous BDNF treatment.

**Cell Viability Assays—** 2.5 x 10^6 NB1643 cells/well were plated in medium (10% FBS/RPMI) containing 5 μM ATRA on poly-lysin-coated 96-well plates (Costar). Final ethanol concentration was 0.1% throughout the experiment. 48 h after ATRA treatment, BDNF (50 ng/ml) and 50 μg/ml streptomyacin (Life Technologies, Inc.) were added in tissue culture plates from Costar. Nuclei were counted as described above. For direct assessment of cell number, triplicate wells were trypsinized, and nuclei were counted and counted as described above.

**Survival Assays in Low Serum—** 2.5 x 10^6 NB1643 cells were plated in 3.5-cm tissue culture wells (Costar) coated with poly-lysin with 5 μM ATRA in 10% FBS/RPMI. Final ethanol concentration was 0.1% in all wells. Medium was changed 48 h later to medium with or without 50 ng/ml BDNF and 5 μM ATRA in 0.1% FBS/RPMI. Triplicate wells were trypsinized, and nuclei were counted as described above. For direct assessment of cell number, triplicate wells were trypsinized, and nuclei were counted and counted as described above.

**Growth Assays in Low Serum—** 2.5 x 10^6 NB1643 cells were plated per well in 6-well tissue culture dishes (3.5-cm-diameter wells) (Costar). Medium was changed 24 h later to medium with or without 5 μM ATRA. For the BDNF growth rate experiments, 2.5 x 10^6 NB1643 cells were plated per well in 6-well tissue culture dishes (3.5-cm-diameter wells) (Costar). Medium was changed 24 h later to medium with or without 5 μM ATRA. After 48 h, BDNF was added (100 ng/ml). Final ethanol concentration was 0.1% in all wells. Triplicate wells were harvested by trypsinization and nuclei were counted as described above. Linear regression and statistical analysis to determine if the dependence of growth rate (slopes) of NB1643 differ significantly were performed using the method described by Zar (26) with Primis software (GraphPad). Analyses were performed with a 5% difference in cell density to allow for growth factor mediated cell growth. Analyses were performed using a 2-tailed t-test assuming unequal variances. The significance level was 0.05.

**Immunoblotting Analysis of TRK and TRKB—** For analysis of TRK and TRKB expression induced by ATRA, 4 x 10^6 NB1643 cells were plated in 10-cm dishes (Corning) and treated with or without 5 μM ATRA 2 days later. Ethanol concentration was 0.1% in all samples. For analysis of 9-cis-RA induction, 4 x 10^6 NB1643 cells were plated with either 5 μM ATRA or 9-cis-RA for 5d. Cells in each dish were lysed in 1 ml of RIPA (30) with fresh 1 mM phenethylisothiourea fluoride (diluted

**BDNF Promotes Chemoprotection of Neuroblastoma Cells—** NB1643 cells were plated at a density of 10^5 per well in 6-well tissue culture dishes (Costar) with or without 5 μM ATRA in 10% FBS/RPMI medium. Final ethanol concentration was 0.1% in all wells. Triplicate wells were harvested by trypsinization and nuclei were counted as described above. Linear regression and statistical analysis to determine if the dependence of growth rate (slopes) of NB1643 differ significantly were performed using the method described by Zar (26) with Primis software (GraphPad). Analyses were performed with a 5% difference in cell density to allow for growth factor mediated cell growth. Analyses were performed using a 2-tailed t-test assuming unequal variances. The significance level was 0.05.
from 100× stock in methanol) at 4°C, and repipetted four times
through a 26 gauge needle. The samples were then taken up
in 30 μl of 2× SDS-polyacrylamide gel electrophoresis sample buffer.
After SDS treatment, the gels were transferred to Immobilon-P (Millipore) using the Milliblot semidry apparatus (Millipore). Immunoblot analysis to detect TRK and TRKB was performed as follows at 22°C. The membrane was blocked for 1 h in 5% milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20). The blot was washed once for 15 min and twice for 5 min with TBST and then incubated with TRK C-14 (1:200) in 1% milk/1% bovine serum albumin in TBST for 1 h. The membrane was then washed as above and incubated with anti-rabbit horse radish peroxidase in 1% milk/1% bovine serum albumin in TBST for 1 h. The membrane was then washed for 15 min and then four times for 5 min in TBST. TRK and TRKB bands were then visualized using chemiluminescent detection with the ECL kit (Amersham Pharmacia Biotech) followed by immunoblotting with TRK C-14 antisera using the methods described above. For TRKB expression, TRKs were immunoprecipitated with TRK C-14 antisera followed by immunoblotting with TRKB-specific antisera 5050 (1:1000) (15, 71) and TRKB antibodies (1:200) (catalog no. 794, Santa Cruz). For analysis of TRK- or TRKB-specific expression, 2 × 10^6 NB1643 cells were plated with either 0 or 5 μM ATRA for 4 days. TRK was immunoprecipitated using 1 μg of TRK-specific antibodies (catalog no. 9142, New England Biolabs) followed by immunoblotting with TRK C-14 antisera using the methods described above. For TRKB expression, TRKs were immunoprecipitated with TRK C-14 antisera followed by immunoblotting with TRKB-specific antisera 5050 (1:1000) (15, 31) and TRKB antibodies (1:200) (catalog no. 794, Santa Cruz).

Kinase Activity of TRK and TRKB—For analysis of NGF-induced autophosphorylation, 4 × 10^5 NB1643 cells were plated in 10-cm dishes (Corning) with or without 5 μM ATRA. 5 days later, cells were treated with 50 ng/ml of NGF for 5 min. For analysis of BDNF-induced autophosphorylation, 5 × 10^5 cells were plated and treated with or without 5 μM ATRA 1 day later. 5 days later, cells were treated with 50 ng/ml BDNF for 5 min. Ethanol concentration was 0.1% in all dishes. Cells were lysed immediately at 4°C, and TRKs were immunoprecipitated, resolved using SDS-polyacrylamide gel electrophoresis, and transferred to Immobilon-P as described above. Immunoblot analysis to detect TRKBs was performed as described above. Immunoblot analysis to detect phosphoryrosine was performed as follows at 22°C. The membrane was blocked for 2 h in 5% bovine serum albumin (Sigma, catalog no. A-6003) in TBST, and then fresh 4G10 anti-phosphotyrosine antibodies (1:5000) (Upstate Biotechnology) were added for 1 h with rotation. The blot was washed once for 15 min and twice for 5 min with TBST and then incubated with anti-mouse horseradish peroxidase in 5% bovine serum albumin (Sigma, catalog no. A-6003) in TBST. The membrane was then washed for 15 min and then four times for 5 min in TBST. TrK and TRKB phosphoryrosine containing protein bands were then visualized using chemiluminescent detection with the ECL kit (Amersham Pharmacia Biotech) and XAR 5 film (Kodak).

Cisplatin Toxicity Assays—2.5 × 10^5 NB1643 cells/well were plated in quadruplicate in medium (10% FBS/RPMI) containing 5 μM ATRA on poly-lysine coated 96 well plates (Costar). Final ethanol concentration was 0.1% through out the experiment. 48 h after ATRA treatment, BDNF (50 ng/ml) and varying concentrations of cisplatin were added in fresh medium containing 5 μM ATRA. Medium containing drug was removed 24 h later and replaced with fresh medium with BDNF (50 ng/ml) and 5 μM ATRA. After 5 days, wells were aspirated, and 1 μl Cisplatin 225. (see below). We next wanted to test whether or not BDNF promoted survival in differentiated NB1643 cells.

RESULTS
A Drug-sensitive Model of NB—As there is evidence that BDNF and its corresponding receptor, TRKB, may have a role in NB exhibiting poor prognosis (23), we wanted to establish a model of NB using a cell line to test the role of BDNF on growth and survival of NB cells. To this end, we characterized a drug-sensitive cell line, NB1643, which has N-MYC amplification and is derived from a stage D NB. The cell line was established from a tumor isolated from a patient who had not yet been treated with chemotherapeutic drugs (32), which may explain the unique drug-sensitive nature of this NB cell line. ATRA induces a striking differentiated phenotype in the NB1643 cell line that is most clearly characterized by extensive neurite outgrowth (Fig. 1). There is also a distinct morphological change in the cell body, which can be described as flattening and elongation. Neurite outgrowth started within a day of ATRA addition and was extensive after 5 days. In addition, NB1643 cells were less aggregated after differentiation induced by ATRA. As might well be expected, differentiation in response to ATRA treatment was accompanied by a reduced rate of cell growth (Fig. 2A). ATRA increased cell doubling time from 2.5 days to 3.5 days. However, there was still cell proliferation, in the presence of serum, even 5 days after ATRA addition. On the other hand, BDNF did not significantly alter the growth rate observed in these differentiated cells in the presence of serum (Fig. 2C). BDNF had no effect on the growth rate of undifferentiated cells (Fig. 2B), which was expected because undifferentiated NB1643 cells do not express TRKB (see below). Next, we wanted to test whether or not BDNF promoted survival in differentiated NB1643 cells.

BDNF Promotes Survival of Differentiated Human NB Cells—BDNF promotes survival of cells under stress resulting from varying levels serum (Fig. 3A). It is interesting that increasing levels of serum in the presence of a constant concentration of BDNF results in increased survival. Perhaps there is synergism between BDNF and a growth factor in serum that promotes survival. Alternatively, perhaps pretreatment with BDNF is required for the effects of a growth factor in serum in these NB cells. Cellular viability was assessed using Calcein AM, which is an established method for measuring cell viability. This assay requires both cellular esterases for conversion of cell-permeable, nonfluorescent, Calcein AM to fluorescent Calcein and intact plasma membranes for retention of the cell im permeant, highly charged, Calcein (33–36). The production of Calcein from Calcein AM by cellular esterases in intact cells was linear with respect to cell number (Fig. 3B). In an independent assay, in which cell number was counted directly, it was also demonstrated that BDNF induced increased survival of NB1643 in low serum (Fig. 3C). Because exogenous BDNF increased cell survival in NB1643 cells, we next wanted to assess the expression of TRKB, the receptor for BDNF.

TRKB and TRK Are Induced by ATRA—It seemed likely that NB cell differentiation may be comparable to NGF-induced differentiation of rat PC12 cells, because the origins of these cell lines are similar. We examined the expression of both TRK and TRKB and their corresponding ligands, NGF and BDNF. ATRA induced expression of TRK 3.2-kilobase mRNA in NB1643 cells (Fig. 4). Although TRK mRNA was not detected...
in the cell prior to ATRA treatment, TRK protein was expressed in NB1643 cells (see Fig. 6A, below). On the other hand, TRKB protein was not detected in these cells prior to ATRA treatment (see Fig. 6A, below). TRKB mRNA expression was induced about 7-, 10-, and 17-fold after 1, 2, and 5 days of ATRA treatment, respectively (Fig. 5A). Clearly, regulation of TRK and TRKB by ATRA occurs at the transcriptional level. However, these experiments do not rule out the possibility of a translational contribution to the regulation of TRK and TRKB levels. The time course of expression of both TRK and TRKB proteins following ATRA treatment was determined using antibodies raised against the C-terminal 14 amino acids of TRK, which recognize TRK and TRKB (Fig. 6A). Interestingly, TRK was induced in 1 day, whereas TRKB was induced after 2 days and TRKB expression was further increased at 5 days following ATRA treatment. Although TRK was expressed before ATRA treatment, ATRA treatment further increased the levels of TRK. On the other hand, TRKB protein was not detected before ATRA treatment in NB1643 cells. Because the antibody raised to the C-terminal 14 amino acids recognizes both TRK and TRKB, the expression of these proteins was examined using specific antibodies. Immunoprecipitation with a TRK-specific antibody followed by immunoblotting with the C-14 TRK antibodies confirmed that TRK was expressed and induced by ATRA in these cells (Fig. 6C). Immunoprecipitation with a TRK C-14 antibodies followed by immunoblotting with TRKB-specific antibodies (13, 31) confirmed that TRKB was expressed and induced by ATRA in these cells (Fig. 6D). In summary, ATRA induces full-length TRKB, which is a receptor for BDNF and a protein-tyrosine kinase.

There is considerable clinical interest in both ATRA and the recently discovered hormone, 9-cis-RA, as biological modifiers (37). Therefore, experiments on the effects of the latter hormone on NB1643 cells were also carried out. 9-cis-RA also causes differentiation and expression of TRK and TRKB in NB1643 cells, although it is less potent than ATRA (Fig. 6B).

**FIG. 2.** Growth of NB1643 cells is decreased by ATRA but unaffected by BDNF. NB1643 were grown in medium containing 0 or 5 μM ATRA and with or without 100 ng/ml BDNF. Cell number was determined by harvesting and counting nuclei. A, ATRA alone reduced the proliferation rate of NB1643 cells (p = 0.002). B, BDNF (100 ng/ml) had no significant effect on the growth rate of undifferentiated cells (p = 0.66). C, BDNF had no significant effect on the growth rate of differentiated cells (p = 0.35), which were cultured for 2 days in 5 μM ATRA and then grown in medium containing 10% FBS and with or without 100 ng/ml BDNF.

**FIG. 3.** BDNF promotes survival of NB1643 cells in low serum conditions. A, BDNF increases cellular viability in low serum conditions. NB1643 cells were cultured for 2 days in 5 μM ATRA. Cells were then cultured in medium containing varying levels of serum (0, 0.1, 0.2, or 0.5% FBS) with or without BDNF (50 ng/ml) for 4 days, after which, cellular viability was assessed by using Calcein AM. B, Calcein AM detection of cells was linear with respect to cell number. NB1643 cells were plated at varying densities and grown overnight. Duplicate wells were either assayed with Calcein AM or nuclei were harvested for direct counting. C, BDNF increased survival of NB1643 in low serum conditions. NB 1643 cells were cultured 2 days in 5 μM ATRA and complete medium and then cultured in 5 μM ATRA in 0.1% FBS/RPMI medium with or without 50 ng/ml BDNF. Cell number was quantitated by preparing and counting nuclei.

**FIG. 4.** BDNF Promotes Chemoprotection of Neuroblastoma Cells

**FIG. 5.** BDNF and NGF Are Expressed in NB1643 Cells—Next, we determined whether BDFN or NGF is expressed in these cells. **BDNF** and, to a lesser extent, **NGF**, are expressed in NB1643 cells (Fig. 5B). ATRA treatment results in a decrease in BDNF mRNA levels. It seems likely that differentiation of NB1643...
cells induced by ATRA or 9-cis-RA may be due to an autocrine loop involving BDNF and TRKB, respectively, and perhaps to a lesser extent, NGF and TRK. The time course of induction of TRKB, as opposed to TRK, is more closely correlated with the time course of phenotypic differentiation. This supports the notion that differentiation may be the result of an autocrine mechanism involving BDNF and TRKB.

**FIG. 4.** ATRA induces expression of TRK mRNA. Northern analysis on 20 μg of total RNA isolated from NB1643 cells lines grown in 0 or 5 μM ATRA for 5 days. The top panel is hybridized with a human TRK 32P-labeled probe, whereas the bottom panel is hybridized with a human 32P-labeled G3PDH probe to verify equivalent loading of RNA samples. The 3.2-kilobase TRK mRNA and G3PDH mRNA are indicated with arrows.

**FIG. 5.** ATRA induces expression of TRKB and BDNF and NGF are expressed in NB1643 cells. Total RNA was isolated from NB1643 cells grown in 0 or 5 μM ATRA for 1, 2, and 5 days. A, RT-PCR analysis for expression of TRKB and G3PDH are shown. Arrows indicate the predicted 371-bp TRKB and 938-bp G3PDH PCR products. B, RT-PCR analysis for expression of BDNF, NGF, and G3PDH are shown. NB1643 cells were treated with 0 or 5 μM ATRA. Arrows indicate the predicted 492-bp BDNF, 432-bp NGF, and 1347-bp transferrin receptor PCR products. 32P-Labeled PCR products were analyzed using a PhosphorImager.
tective effect against cisplatin treatment. This is not surprising considering the sequence homology and functional similarities between the TRK family members.

**DISCUSSION**

The data presented here indicate that BDNF is a trophic factor for NB cells, at least in this model of NB. Perhaps more significantly, BDNF protects these NB cells from cisplatin, which is a drug that damages DNA. We tested this hypothesis, in part, because of the extensive data for the function of neurotrophins in models addressing neurodegenerative and other trauma-induced diseases of the nervous system. These studies range from findings that BDNF and TRKB are induced in models of stress to data from an *in vivo* model suggesting that TRKB is required for survival of damaged neurons. In models of neuronal damage in the hippocampus, TRKB and BDNF expression is increased following cellular insult (39). Hippocampal kindling, which causes seizures, leads to a rapid transient increase of TRKB mRNA and protein in the hippocampus. Levels of TRK or TRKC were not altered by kindling. Other conditions, such as ischemia and hypoglycemic coma, resulted in a similar increase in TRKB mRNA in the dentate gyrus. These treatments also left TRK and TRKC mRNAs levels unaltered. Following exposure to kainate, which also induces seizures, TrkB mRNA increased in dentate granule cell and CA1 pyramidal cell layers of the adult hippocampus (40). A similar increase in BDNF mRNA was also observed following kainate treatment in the pyramidal and granule cell regions. Spinal cord lesions in rats, which do allow some axonal regrowth, lead to increased levels of TRKB, suggesting a role for TRKB in axonal sprouting in injured spinal cords (41, 42). These results suggest that up-regulation of TRKB and BDNF may form an autocrine loop promoting cell survival. This may be a protective response to neuronal insult.

There are data that support a target-derived mechanism for trophic support for neurons provided by neurotrophins. However, in experiments designed to test an autocrine role for BDNF using BDNF and TRKB antisense oligonucleotides resulted in increased death of sensory neurons (43). This provides direct evidence that BDNF may function in an autocrine fashion in some neuronal populations. BDNF protects rat hippocampal, septal, and cortical cultured neurons against metabolic and excitotoxic insults (44). Activation of voltage-sensitive calcium channels results in increased survival of cultured rat embryonic cortical neurons (45). It was shown, using antibodies to BDNF, that BDNF is required for this observed increase in survival. Taken together, these studies suggest that BDNF...
grown for 5 days in 0 or 5 μM BDNF, for 5 min. Immunoprecipitation of TRKs, followed by anti-phosphotyrosine immunoblotting indicates that gp145TRK, but not gp140TRK, is phosphorylated on tyrosine in response to BDNF.

**Fig. 7.** ATRA induces TRK and TRKB, which is competent for NGF- and BDNF-induced autophosphorylation. A, NB1643 cells grown for 5 days in 0 or 5 μM ATRA were treated with 50 ng/ml NGF for 5 min. Immunoprecipitation of TRKs, followed by anti-phosphotyrosine and anti-TRKs immunoblotting indicates that gp140\(^{TRK}\), but not gp145\(^{TRK}\), is phosphorylated on tyrosine in response to NGF. B, NB1643 cells grown for 5 days in 0 or 5 μM ATRA were treated with 0 or 50 ng/ml BDNF for 5 min. Immunoprecipitation of TRKs, followed by antiphosphotyrosine immunoblotting indicates that gp145\(^{TRK}\), but not gp140\(^{TRK}\), is phosphorylated on tyrosine in response to BDNF.

**Fig. 8.** BDNF and NGF induce FOS expression. NB1643 cells were treated with 5 μM ATRA. After 5 days, cells were treated with either BDNF or NGF (100 ng/ml) for 30 min and then analyzed for FOS and G3PDH expression using RT-PCR. \(^{33}P\)-Labeled PCR products were analyzed using a PhosphorImager. Arrows indicate the predicted 482-bp FOS and 983-bp G3PDH PCR products.

may have a role in protection of mature neurons from cellular insults. It seemed plausible, based on these studies, that neurotrophins may protect cells from a plethora of stressful conditions, which led us to investigate and demonstrate that BDNF induces drug resistance in an NB cell line.

BDNF and NGF induce about a 2-fold shift in the EC\(_{50}\) values for cisplatin toxicity in NB1643 cells, indicating that the factors do induce drug resistance. The complete survival observed in the presence of neurotrophins is even greater, as it seems to derive from a combined effect on survival and drug resistance by these factors. To address the clinical significance of these data, we have contrasted them with clinical pharmacokinetic data on serum cisplatin levels. Delivery of cisplatin by infusion of 90 mg/m\(^2\) over 6 h resulted in serum levels between 1–4 μg/ml that declined rapidly (46). In another study contrasting infusion of 100 mg/m\(^2\) cisplatin over varying periods, 2–7 h, peak cisplatin serum levels were in the 1–5 μg/ml range (47). Shorter infusion periods resulted in predictably higher peak serum concentrations of cisplatin. Infusion over the longest period, 7 h, resulted in serum cisplatin concentrations of 1 μg/ml with a several-hour plateau. Cisplatin levels declined rapidly following infusion in every case. This concentration range (1–5 μg/ml) is comparable to the effective concentration range of cisplatin presented in Fig. 10. If the sensitivity of NB cells to cisplatin is altered in this concentration range, it may allow survival of some NB cells. Clinically relevant drug resistance may not require order of magnitude shifts, but rather just severalfold shifts in the concentrations of drugs required to induce cell death. Cells need only survive the achievable therapeutic dose to have effective drug resistance.

In vitro selection of two different NB cell lines using escalating doses of cisplatin resulted in resistant cell lines (48). The resistant cell lines, IMR and SK-N-SH, were 6.6- and 3.8-fold, respectively, more cisplatin-resistant than the parental NB lines. The parent SK-N-SH line was more resistant originally than the parent IMR line. Mechanisms of resistance in these cell lines were not established, although the involvement of enhanced DNA repair is discussed. These data also underscore that shifts in the cisplatin concentrations required to induce cell death resulting in drug resistance may be small.

The findings presented here and previously (49, 50) underscore the importance of an autocrine loop involving TRKB and BDNF in differentiation of NB cells. In SH-SY5Y NB cells, TRKB is induced by ATRA and, here, treatment with exogenous BDNF causes differentiation. ATRA treatment of KCNR cells, which already express BDNF, induces TRKB expression and subsequent differentiation. However, in 15N NB cells, ATRA induces only a truncated TRKB receptor and although these cells express BDNF, ATRA does not induce differentiation. These reports, and the data reported here, provide evidence that ATRA induces differentiation by creating an autocrine loop with BDNF and TRKB.

It seems plausible that ATRA, by regulating expression of TRK and TRKB, may be required for normal development or maintenance of sympathetic neurons. ATRA induces NGF-dependent survival in embryonic day 7 sympathetic neurons from chick (51). ATRA treatment of embryonic chick sympathetic neurons leads to increased levels of TRK, resulting in NGF-dependent survival (52). This response is mediated by the α-retinoic acid receptor. Although not conclusive, these studies implicate ATRA in a developmental role during sympathetic neuronal development. A reasonable hypothesis is that ATRA, or other retinoids, may be required for induction of TRK family members during development of sympathetic neurons. Experiments with a panel of retinoic acid receptor selective retinoids...
has provided evidence that 3 distinct retinoic acid receptor/retinoid X receptor heterodimers may be involved in mediating the effects of ATRA and 9-cis-RA in NB cells (53).

In contrast to TRKB expression in NB, there are findings that TRK expression is correlated with a favorable prognosis in NB (54–56). In addition to a correlation between TRK expression and stage, there is an inverse relationship between TRK and N-MYC expression in NB (56–58). N-MYC amplification is a well established negative prognostic indicator for NB (59). Although the evidence is compelling that TRK expression is a positive prognostic indicator for NB, the reason TRK expression is correlated with prognosis and stage is unknown. One explanation for this correlation between TRK expression and a favorable outcome in NB is that TRK might cause differentiation and regression of low stage NB. Alternatively, low stage NB with favorable prognosis may require TRK for survival, whereas high stage NB may not. In contrast, in this study, we have examined neurotrophin function in a cell line derived from a high stage NB tumor.

Investigations of some NB cell lines have suggested that restoration of TRK expression, either by transfection or up-regulation, leads to differentiation in response to NGF. Transfection of TRK cDNA into HTLA230 cells followed by NGF treatment resulted in growth arrest and differentiation, as well as N-MYC down-regulation (60). This cell line, HTLA230, was isolated from a patient with stage IV disease and notably lacks expression of TRK. In SH-SY5Y, NGF in conjunction with aphidicolin, an inhibitor of DNA polymerase α, induce differentiation (61).

Differentiation is accompanied by up-regulation of TRK and down-regulation of c-MYC. In another report on the same cell line, the role of TRK was tested directly by exogenous expression of TRK (62). SH-SY5Y cells engineered to express TRK differentiate in response to exogenous NGF treatment. In contrast to the previous report (61), treatment of these cells with 12-O-tetradecanoylphorbol-13-acetate induced endogenous TRK expression, but the cells did not differentiate in response to NGF treatment. It is possible that TRK expression induced by 12-O-tetradecanoylphorbol-13-acetate is not sufficient for the sustained response necessary for NB differentiation. Alternatively, there may be defects in the endogenous TRK receptor in these cells. Regardless, these data do suggest that TRK receptor causes differentiation and growth arrest of NB. This could explain TRK expression in NB with favorable prognosis and, likewise, how decreased or loss of TRK expression or defects in signaling events downstream of TRK could cause NB with poor prognosis.

Whether BDNF requires common or independent signaling pathways for promoting survival or chemoresistance remains unknown. Neurotrophins, via TRKs, activate at least three signaling pathways (RAS, PI3K, and PLC-γ1), and perhaps a fourth pathway requiring SNT, which may be identical to FRS2 (63, 80). Ligand-induced dimerization (38) and autophosphorylation (38) forms binding sites for protein substrates containing SH2 (13, 64) and PTB (65) domains. RAS is activated in a pathway requiring SHC association with TRK (66). Neurotrophins induce PLC-γ1 association with TrkB (13), and PLC-γ1 is phosphorylated and activated (13, 64, 67). PI3K is definitively activated by TRK. However, it is likely that PI3K does not form a ligand-dependent association complex with TRK (68). There is, however, a report that PI3K associates with phosphorylated tyrosine 751 of TRK (69). However, it is more probable that PI3K is activated indirectly by TRK through another mechanism, such as another adapter phosphoprotein or RAS. RAS can activate PI3K (70). There are also recent data indicating that BDNF may activate other signaling pathways involving rAPS and SH2-B (40).

The question of which signaling pathways activated by TRKs
are required for NB cell differentiation and survival is not completely resolved. PC12 cells differentiate in response to NGF (71). RA activation is required for PC12 cell differentiation. SHC, and to some extent PLC-γ1, may be required for differentiation of PC12 cells (66), although other data indicate redundant roles for PLC-γ1 and RA in ERK1 activation (72). PC12 cells expressing a point mutant of TRK, which cannot activate PLC-γ1, still extend neurites in response to NGF (73). There are data suggesting that a fourth pathway may be activated by TRK involving SNT (74), which may be required for neuritogenesis, but not survival (75). The role that SNT, which may be identical to FRS2 (63, 80), has in both differentiation and survival in response to neurotrophins is not understood. However, FRS2 has been shown to be required for differentiation of PC12 cells in response to FGF (76). A role for PI3K in differentiation has also been suggested. Wortmannin, an inhibitor of PI3K, has been reported to block NGF-induced neurite outgrowth in PC12 cells (77). It is likely that the primary role for neurotrophins during development and, perhaps, in the mature adult is as trophic or survival factors. In PC12 cells, NGF prevents cell death in serum-free medium (71). PI3K signaling may be required for NGF-induced survival of PC12 cells (78). It will prove interesting to examine the role that PI3K may have in chemoprotection. It is now apparent that investigations into the role that SNT or FRS2 has in neurons will also be required to resolve the signaling pathways required for the phenotypic effects of neurotrophins.

In conclusion, BDNF promotes survival and chemoprotection against cisplatin in differentiated NB1643 cells. Experiments assessing whether BDNF affords protection from other DNA-damaging agents, such as topoisomerase inhibitors and ionizing radiation, are underway. This will suggest whether BDNF antagonizes a general apoptotic response to DNA damage or affects just specific classes of DNA-damaging agents. BDNF has been shown to protect another NB cell line, 15N, which was engineered to express TRKB, from vinblastine (79). Vinblastine promotes tubulin depolymerization. In NB1643 cells, ATRA induces a phenotypic differentiation that is accompanied by increased expression of functional TRKB receptor protein-tyrosine kinase. Because BDNF is expressed in these cells, an autocrine loop is formed involving BDNF and TRKB that is likely involved in differentiation of these cells. Treatment with exogenous BDNF promotes even greater neurite extension. It will be of great interest to delineate the signaling pathways required for survival and drug resistance in NB wells, both at the membrane receptor level and further downstream in other pathways controlling cell growth and apoptosis.

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