Expression of *trkA* cDNA in Neuroblastomas Mediates Differentiation In Vitro and In Vivo

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Received 18 May 1993/Returned for modification 5 July 1993/Accepted 18 September 1993

The human *trkA* cDNA was transfected into a malignant human neuroblastoma (NB) cell line (HTLA230) to investigate its role in NB growth and differentiation. This cell line lacks expression of both endogenous *trkA* and *gp75^{NGFR}* genes. Transfectants expressing the *trkA* mRNA and surface-bound receptors transcriptionally activate immediate-early genes (*c-fos*, *c-jun*, and *jun-B*) following nerve growth factor (NGF) stimulation. NGF treatment induces growth arrest as well as down-regulation of the amplified *N-myc* oncogene. Genes selectively expressed in mature neurons (SCG-10, *ret* proto-oncogene, GAP-43, etc.) are transcriptionally activated, and neurite outgrowth further demonstrates differentiation of transfectants following NGF stimulation. *trkA*-expressing NB cells remain tumorigenic in nude mice; however, subcutaneous treatment of tumor-bearing mice with NGF induces Schwannian and neuronal cell differentiation similar to the induction seen in human ganglioneuroblastomas. Thus, *trkA* expression in HTLA230 cells is sufficient to generate a functional NGF receptor complex that leads to growth-arrested and differentiated NB cells in vitro and in vivo in the presence of NGF. Hence, NGF may play a crucial role in NB cell differentiation and regression in vivo.

Nerve growth factor (NGF) is a member of the growing family of neurotrophic factors (neurotrophins) and is vital for growth, differentiation, and survival of sympathetic and neural crest-derived sensory neurons (5). The growth factor is also important in the development and maintenance of specific types of neurons of the central nervous system (4). Several other NGF-related neurotrophins, such as BDNF, NT3, and NT4/5, have recently been isolated, and their role in neuronal cell growth and differentiation is being studied. A family of cell surface receptors (encoded by *trkA*, *trkB*, and *trkC*) to which these neurotrophic factors bind has been identified, and their respective genes have been cloned. The various members of the *trk* gene family have preferred binding associations with specific neurotrophins, thus controlling the specificity of signalling. The *trkA* receptor preferentially interacts with NGF, while *trkB* shows equal functional binding associations with BDNF and NT4/5. The *trkC* receptor strongly associates with NT3 (20). Promiscuous receptor-ligand interactions have also been noticed (5). These data have predominantly been generated with NIH 3T3 cells lacking receptors for neurotrophins and transfected with the various *trk* genes. Membrane-associated *trk* receptors rapidly autophosphorylate, mediated by a tyrosine kinase activity, following ligand binding (27). Cell growth and transformation of *trkA*-, *trkB*-, or *trkC*-expressing NIH 3T3 cells occurs upon continuous stimulation of the receptor cascade with the preferred ligand (reference 20 and references therein). Thus, *trk* receptors utilize a signalling mechanism similar to that demonstrated with classical growth factor receptors (48).

However, a low-affinity NGF-binding receptor (*gp75^{NGFR}*) that binds all members of neurotrophins has been known for some time (24, 45). Although there seems to be a good general consensus that *trk* receptors mediate the functional

cell signal of neurotrophins, there are conflicting views as to the role of the *gp75^{NGFR}* in the signal pathway. The gene encoding the *gp75^{NGFR}* has been cloned, and ectopic expression in fibroblasts generated only low-affinity binding sites for NGF (24, 44). On the other hand, expression of *gp75^{NGFR}* cDNA in human neuroblastoma (NB) cells or mutant PC12 cells restored responsiveness to NGF in these transfectants, suggesting the requirement of additional neuronal components necessary for NGF signalling through *gp75^{NGFR}* (16, 37). In some reports, it has been suggested that the presence of both the *gp75^{NGFR}* and the *trkA* molecules is required for an NGF-mediated response (15), while other data indicate a *trkA*-mediated signalling in the absence of *gp75^{NGFR}* (17, 27, 40, 60). These latter data are further supported with observations made in mice carrying a null mutation for the *gp75^{NGFR}* gene (30). These animals showed only marginal alterations and dysfunctions of the peripheral sensory nervous system, not of sympathetic neurons. Thus, the role of each receptor type in the NGF response is not entirely resolved. We therefore have investigated the role of *trkA* in neuronal cell growth and differentiation in vitro and in vivo, using a human NB cell line lacking expression of both *gp75^{NGFR}* and *trkA* genes.

NB is a childhood tumor of neural crest origin. The tumor often shows various degrees of cellular differentiation, and spontaneous regression occurs in a subset (stage IVS) of NB (51). The molecular mechanisms controlling these processes are unknown; however, one could speculate that neurotrophic factors and their respective signal pathways may play an important role. We and others demonstrated that *trkA* expression is high in most of the differentiated primary ganglioneuroblastomas and in all stage IVS tumors, further supporting this hypothesis (41, 55). A statistically significant correlation of *trkA* gene expression and patient survival was also determined, substantiating the importance of neurotrophic receptors and their ligands in the pathogenesis of NB. The present study demonstrates that transfection of the *trkA*

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cDNA into NB reconstitutes a functional NGF-mediated signal cascade that leads to growth arrested and differentiated NB cells in the absence of gp75^{NGFR}. We further demonstrate that nude mouse tumors expressing the *trkA* gene differentiate into Schwannian and neuronal components upon treatment with NGF similar to that seen in primary human ganglioneuroblastoma.

MATERIALS AND METHODS

Cell culture. HTLA230 cells were isolated from a patient with stage IV disease. The cell line has extensively been characterized (39). Cells were grown in Dulbecco's modified Eagle's medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum, penicillin-streptomycin, and glutamine (all from GIBCO) as previously described (38). Cells were transfected by the calcium phosphate precipitation method and selected in Geneticin (G418; 1 mg/ml; GIBCO). Extracellular matrices (ECM) from R₂₂ClF cells were produced as previously described (38) and used as the cell substrate. Mouse 2.5S NGF was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Autoradiographic analysis of [³H]thymidine ([³H]TdR; 1 µCi/ml; Amersham Corp., Arlington Heights, Ill.) was performed as previously described (39).

Northern (RNA) blot analysis. Cells were grown under the appropriate experimental conditions, washed twice with ice-cold phosphate-buffered saline (PBS), and harvested for RNA extraction as described by Chirgwin et al. (6). Northern blots prepared with 20 µg of RNA per lane unless otherwise stated were hybridized with the following probes: human proto-oncogene *trk* (36), human low-affinity NGFR (24), *N-myc* (49), *v-fos* (8), *c-jun* (14), *jun-B* (42), 68-kDa neurofilament (NF-L) (19), SCG-10 (54), microtubule-associated protein 2 (9), GAP-43 (25), proto-oncogene *ret* (56), *smg p25A* (47), IF-clone 73 (peripherin) (31), S100α (28), and S100β (29). Rehybridization with a human α-tubulin cDNA clone, pkα-1 (7), or a human glyceraldehyde 3-phosphate dehydrogenase cDNA clone, pHcGAP (34), was carried out to normalize RNA loading. Blots were washed at 60°C in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Western blot (immunoblot) analysis. Western blotting was performed according to standard protocols (59). Cells were cultured at equal cell density for 24 h prior to NGF stimulation for 5 min and harvested in radioimmunoprecipitation assay buffer for immunoprecipitation. Polyacrylamide gels were loaded with immunoprecipitation samples from 25 µg of cell lysates per lane, and proteins were transferred to nitrocellulose filters and blotted with an antiphosphotyrosine antibody (Upstate Biotechnology, Inc., Lake Placid, N.Y.). Immunoprecipitation using the anti-*trkA* antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) was done according to the manufacturer's instructions. The enhanced chemiluminescence system (Amersham) was used to visualize the Western blots.

Immunohistochemistry and FACS analysis. Cultured cells were rinsed twice in PBS, fixed in situ in a 1:1 mixture of methanol-ethanol, and washed three times with cold PBS. Cells were then stained with a mouse monoclonal anti-human *trkA* antibody kindly provided by K. Eager (10) and incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Cappel, Organon Teknika, Durham, N.C.). For fluorescence-activated cell sorting (FACS) analysis, cells were harvested in PBS containing 5

FIG. 1. Northern blot analysis of *trkA*-transfected HTLA230 clones. Total cellular RNA was isolated from individual clones treated with NGF (100 ng/ml) for 40 min and hybridized with probes for *trkA* and *c-fos* genes.

mM EDTA and subsequently stained with anti-*trkA* antibody as previously described (37). Expression of S100α and S100β subunits in tumors was analyzed with polyclonal rabbit antisera and visualized with peroxidase-antiperoxidase (57).

Nude mice. Cultured cells of the different cell lines were injected (5×10^6 cells per injection) into the flanks of Swiss BALB/c nude mice, and tumors were analyzed at indicated times. Tumor-bearing mice were daily injected subcutaneously with NGF (20 µg) for 6 consecutive days according to a protocol described by Yaeger et al. (61). The mice were sacrificed 24 h after the last administration, and the histopathology of the tumor tissue was analyzed.

RESULTS

We have previously demonstrated that the human NB cell line HTLA230, derived from a stage IV tumor, lacks expression of gp75^{NGFR} (37). We have recently confirmed this observation by reverse transcription-polymerase chain reaction. We further showed that HTLA230 cells do not respond functionally to NGF treatment and thus lack high-affinity receptors for NGF as well (37). We define a functional NGF response in NB cells as the activation of immediate-early genes (*c-fos*) and neurite outgrowth (52). We transfected HTLA230 cells with the *trkA* expression vector pDM69 (36) to investigate the role of *trkA* in NB cell growth and differentiation in vitro and in vivo. Several neomycin (G418)-resistant clones were screened for *trkA* gene expression and the ability to activate *c-fos* gene transcription in the presence of NGF (Fig. 1). Only clones expressing the transfected *trkA* gene showed an immediate-early response, while several G418-resistant clones lacking a *trkA* message did not activate the *c-fos* gene. These data further show the presence of all downstream components necessary for an NGF signal mediation to the cell nucleus in HTLA230 cells. All three *trkA*-expressing clones showed similar biological responses to NGF, and clone 18-10 was subsequently selected for this study.

Surface expression of *trkA* in mock-transfected HTLA230 cells and *TrkA*-transfected cells with use of an anti-*trkA* monoclonal antibody was determined by FACS analysis (Fig. 2). The fluorescence histograms of mock transfectants stained with anti-*trkA* and FITC-conjugated secondary antibodies or with secondary antibody alone were indistinguishable (data not shown). The fluorescence histogram obtained

FIG. 2. FACS analysis of *trkA* transfectants. Transfectants were stained with anti-human *trkA* antibody or the FITC-conjugated secondary antibody (black area under the curve) alone. *trkA*-expressing cells show heterogeneous surface staining ranging from near background to bright fluorescence. Nearly 50% of transfectants showed greater than background fluorescence. The left panel represents the distribution of forward light scatter (y axis) versus fluorescence intensity (x axis), and the circled area displays the analyzed cell population.

with *trkA* transfectants incubated with the FITC-conjugated secondary antibody alone (black area under the curve) demonstrated only background fluorescence, while a broad fluorescence histogram which is shifted to the right of the diagram was observed when cells were stained with anti-*trkA* and FITC-conjugated secondary antibodies. This analysis demonstrated that the *trkA* molecules are surface bound and that transfectants show very heterogeneous *trkA* expression similar to that found with other cell surface proteins following gene transfer (37).

The *trkA* receptor belongs to the family of transmembrane tyrosine kinases which autophosphorylate following ligand binding. To investigate receptor activation in HTLA230-neo and 18-10 cells, we stimulated them with NGF for 5 min and then immunoprecipitated cellular lysates with the anti-*trkA* antibody. Western blots were analyzed with an antiphospho-

tyrosine antibody (Fig. 3A). Unstimulated and NGF-stimulated HTLA230-neo cells did not show any detectable *trkA* protein. In contrast, a 140-kDa protein could be precipitated from 18-10 cells, and this immunoprecipitation was abolished in the presence of the control peptide to which the anti-*trkA* antibody was generated. The *trkA* protein was present in a ligand independently phosphorylated form in resting 18-10 cells as has previously been described (32), and an enhanced level of phosphorylation was seen in stimulated transfectants. We concluded that mock-transfected HTLA230-neo cells did not express the *trkA* gene and that 18-10 cells expressed a membrane protein with the characteristics of the *trkA* receptor.

NGF-mediated differentiation of neuronal cells is preceded by activation of immediate-early genes (13). Transfectants were stimulated with NGF for 40 min in the presence or

FIG. 3. Western blot and Northern blot analyses of NGF-stimulated cells. (A) Immunoprecipitation of cellular lysates of unstimulated or NGF-stimulated HTLA230-neo and 18-10 cells, using the anti-*trkA* antibody. Mock-transfected HTLA230-neo cells did not show detectable *trkA* protein, while 18-10 cells contained a 140-kDa protein with the characteristics of *trkA*. The immunoprecipitation was abolished by preincubation of the antibody with control peptide. (B) Northern blot analysis of immediate-early gene activation following NGF treatment. Cells were treated with NGF (100 ng/ml) in the absence or presence of K252a (200 nM), and total RNA was hybridized with either the *c-fos* or α -tubulin probe. NGF treatment of transfectants activated *c-fos* gene transcription; however, pretreatment for 18 h with K252a completely abolished the onset of *c-fos* gene expression. Only 5 μ g of RNA from PC12 cells was loaded. RNA from untreated and treated transfectants was also hybridized with probes for *c-jun* and *jun-B* (C). Both genes were transcriptionally activated in *trkA* transfectants.

FIG. 4. Analysis of growth and *N-myc* gene expression in NGF-treated transfectants. (A) Mock-transfected (squares) and *trkA*-expressing (triangles) cells were grown on the R_{22} CIF matrix in the absence (closed symbols) or presence (open symbols) of NGF (100 ng/ml) in duplicate cultures, and cell numbers were determined at the indicated time intervals. Rapid cell growth of mock-transfected HTLA230 cells occurred independent of the presence or absence of NGF. *trkA*-expressing cells showed substantial cell growth in the absence of NGF; however, cells were essentially growth arrested in the presence of NGF. The results shown are data from two independent experiments. The means represents less than 10% variation. (B) *N-myc* gene expression was analyzed by Northern blotting following NGF treatment. High *N-myc* (MYCN) gene expression was found in untreated transfectants and parental HTLA230 cells; however, NGF-treated cells showed dramatic down-regulation of the *N-myc* oncogene. The human melanoma cell line A875 (11) was used as negative control RNA. The blot was subsequently stripped and rehybridized with a probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to test for RNA loading. d, days.

absence of K252a, a selective inhibitor of the tyrosine protein kinase activity of the *trk* family (58). The *c-fos* gene was activated in *trkA* transfectants similarly to what was seen with PC12 cells; however, transcription activation was completely abolished in transfectants pretreated with K252a, demonstrating that the growth factor response was mediated by the expressed *trkA* gene (Fig. 3B). We also hybridized total RNA from NGF-treated transfectants with probes for *c-jun* and *jun-B*, to analyze the expression pattern of other immediate-early genes mediated by *trkA* in the absence of $gp75^{NGFR}$ (Fig. 3C). Both genes were transcriptionally activated in *trkA*-expressing NB cells following NGF treatment, while high levels of only *jun-B*, not *c-jun*, were found in treated PC12 cells, consistent with previous observations (21). Thus, surface-bound *trkA* mediates an NGF signal that activates a panel of immediate-early genes in the absence of $gp75^{NGFR}$.

We next investigated the effect of NGF on cell growth in mock-transfected and *trkA*-expressing cells (Fig. 4A). We used an ECM (R_{22} CIF) as cell substrate for these experiments, since NB cells easily detach from plastic and hence the ECM substantially improved the accuracy and reproducibility of cell determinations (38). Mock-transfected HTLA230 cells showed rapid cell growth, independent of the presence or absence of NGF. Similarly, *trkA* transfectants grown in the absence of NGF showed substantial cell growth, although a somewhat lower level of cell density was reached. However, *trkA* transfectants grown in the presence of NGF showed a dramatic reduction in cell growth and only minor residual cell proliferation continued, possibly because of the presence of NGF-unresponsive transfectants. These data clearly demonstrate that *trkA* expression in NB cells reconstitutes a NGF signal pathway that leads to growth arrest and possibly postmitotic tumor cells in the absence of $gp75^{NGFR}$.

Aggressive NB generally show amplification and overexpression of the *N-myc* oncogene (12, 50). We have previously demonstrated that parental HTLA230 cells have high *N-myc* gene amplification and expression (39). *N-myc* gene expression in *trkA*-transfected cells following NGF treatment was investigated by Northern blot analysis in order to determine a link between oncogene expression and growth arrest (Fig. 4B). Untreated transfectants show a high level, while NGF-treated transfectants showed a dramatically reduced level, of *N-myc* expression 3 days after NGF stimulation. The *N-myc* mRNA level subsequently remained low throughout the experiment. This reduction of *N-myc* gene expression preceded the pattern of growth arrest observed by the growth analysis (Fig. 4A). The residual level of *N-myc* gene expression possibly reflects proliferation of NGF-unresponsive transfectants. To substantiate this observation, we analyzed DNA synthesis in NGF-treated transfectants by using an overnight pulse of [3 H]TdR (Fig. 5A and B). Autoradiographic analysis revealed that NGF-unresponsive and undifferentiated NB cells had high nuclear labeling, while the majority of differentiated cells showed no incorporation of the label. Few differentiated NB cells, following a 12-h period of labeling with [3 H]TdR, showed residual DNA synthesis. Similar observations have been obtained with growth-arrested PC12 cells following NGF stimulation (18).

trkA transfectants were grown on the R_{22} CIF matrix to further elaborate a link between NB growth arrest and cell differentiation. The R_{22} CIF matrix was previously shown by us to promote cell differentiation of various neuronal and nonneuronal cell types (38). Transfectants were seeded at low cell density, grown for 2 days in regular growth medium, and treated for 8 days with NGF (Fig. 5C and D). Untreated *trkA* transfectants showed a typical flat and undifferentiated morphology similar to that seen with parental and mock-transfected cells (Fig. 5C) (39). However, *trkA*-expressing

FIG. 5. Autoradiographic, morphological, and immunohistochemical analysis of NGF-treated *trkA* transfectants. Incorporation of [3 H]TdR ($1 \mu\text{Ci/ml}$) prior to fixation into NGF-treated transfectants was analyzed by autoradiography (A and B). Differentiated transfectants show only occasionally labeled nuclei, while NGF-nonresponsive and undifferentiated cells (arrowheads) show high incorporation of the label. (A, phase contrast; B, bright-field micrograph; magnification, $\times 87$). Transfectants were grown on the R_{22} C1F matrix for 7 days in the absence (C) or presence (D) of 100 ng of NGF per ml. Untreated cells show a typical flat cell morphology with short cellular processes. In contrast, NGF-treated transfectants formed an extensive network of neurites with many varicosities and arborizations. The R_{22} C1F matrix can be seen as fine network of fibrils in the background. (Magnification, $\times 100$.) NGF-treated cells were stained with anti-*trkA* antibody to investigate *trkA* expression (E). The cell bodies and neurites of differentiated cells were brightly stained. Note that undifferentiated cells had no or only background fluorescence. (Magnification, $\times 372$.)

cells showed a dramatic morphological change in the presence of NGF (Fig. 5D). Cells formed an extensive network of neurites within 3 to 5 days of treatment. Small round cell bodies of morphologically differentiated cells extended numerous neurites typical of mature neurons grown in vitro. The neurites had numerous varicosities and small arboriza-

tions. Only very few cells with an undifferentiated flat cell morphology were present in NGF-treated cultures, demonstrating that most of the *trkA* transfectants responded to NGF. The morphological analysis was performed with NGF at 100 ng/ml, similar to concentrations used by others (16, 32, 33), although drastic morphological changes could also be observed with picomolar concentrations of NGF.

We stained cultures with the anti-*trkA* antibody in order to investigate *trkA* expression in undifferentiated and differentiated 18-10 cells following NGF treatment (Fig. 5E). Morphologically differentiated cells showed intense surface staining, which highlighted their cell bodies and neurites, while undifferentiated cells demonstrated weak or no staining similar to that seen following incubation with the secondary antibody alone.

Induction of morphological differentiation in cultured NB cells has been achieved with various biological compounds; however, phenotypic changes are not necessarily accompanied by the onset of gene expression characteristic for mature neuronal cells (39). We therefore analyzed the expression pattern of various marker genes associated with neuronal differentiation following NGF treatment in *trkA* transfectants (Fig. 6). The SCG-10 gene, originally isolated from a rat superior cervical ganglion (54), showed a low mRNA level in untreated transfectants; however, NGF treatment dramatically increased the transcript level (Fig. 6A). SCG-10 gene activation in *trkA* gene-expressing cells also required long-term NGF treatment, consistent with previous observations (3). Expression of the *ret* proto-oncogene, a cell surface receptor with yet an unknown ligand, was also investigated, since expression of this gene was found to correlate with the differentiated state of neural crest-derived tumors (56). The *ret* proto-oncogene message was undetectable in untreated transfectants; however, a typical multiple transcript expression pattern was observed in NGF-treated *trkA*-expressing NB cells (Fig. 6B). Additional marker genes of neuronal differentiation were investigated by Northern blotting (data not shown). The neurofilament NF-L gene was present as 3.5- and 2.3-kb transcripts in untreated transfectants, but differential up-regulation of the 2.3-kb message was seen after 4 days of NGF treatment. Similarly, GAP-43 (25), IF-clone 73 (peripherin) (31), microtubule-associated protein 2 (9), and the *smg* p25A gene (47) also show increased levels of mRNA in NGF-treated cells. Only a low level of S100 β mRNA was detected in untreated transfectants, and neither the S100 α nor the S100 β gene was transcriptionally activated following treatment. No gp75^{NGFR} message could be detected before and after NGF stimulation. Thus, *trkA*-expressing NB cells show growth arrest, morphological changes, and activation of genes generally associated with terminal neuronal differentiation upon NGF treatment.

Patients with stage IVS NB often show spontaneous differentiation and regression of the tumor; however, mechanisms controlling these events are unknown. We postulate that a neurotrophic factor-mediated signal cascade may be one mechanism regulating NB differentiation in vivo. We tested this hypothesis with *trkA*-expressing NB cells injected into the flanks of Swiss BALB/c nude mice. Tumors developed with equal frequency from the mock-transfected cell line (six of six mice) and the transfected 18-10 cell line (five of six mice), indicating that *trkA* expression per se does not change the tumorigenic property of NB cells. Both malignancies were very hemorrhagic, and tissues were composed of densely packed, undifferentiated neuroblasts. Both tumor tissues showed a high number of mitotic figures and karyor-

rhesis (Fig. 7A). We reestablished primary cultures from subcutaneous tumors of *trkA* transfectants, and onset of *c-fos* gene transcription and neurite outgrowth demonstrated the presence of a functional NGF cascade (data not shown). The in vivo response to NGF of tumors expressing *trkA* was investigated by using a previously established protocol (61). Histopathological analysis of *trkA*-expressing tumors following NGF treatment (Fig. 7B) showed a morphology dramatically different from that seen with untreated (Fig. 7A) or treated parental cell-derived tumors. Very few mitotic figures could be identified following NGF stimulation. The tumor tissue was composed of numerous differentiated neurons characterized by their large cell bodies with eosinophilic cytoplasm and peripherally located, enlarged nuclei. These differentiated neurons were scattered within a well-developed neurofibrous stroma characterized by the presence of extensive Schwannian and other support elements. Such stromal components were completely missing in parental and untreated tumors. The presence of Schwannian components was further substantiated by the immunoreactivity of the tumor with anti-S100 α and anti-S100 β antibodies (Fig. 7C to F). Mild to moderate immunoreactivity with the anti-S100 α antibody and strong staining with the anti-S100 β antibody were observed in treated tumors, while only background staining was present with untreated (Fig. 7C and E) or parental tumors. Electron microscopy of NGF-treated tumors also revealed the presence of an extensive network of neuronal and Schwannian cell processes, a further indication of bimodal differentiation in NGF-responsive tumors (data not shown). In one nude mouse experiment in which established subcutaneous tumors were treated for 7 rather than 6 days, we observed central necrosis of the tumor. Overall, the histopathology of NGF-treated *trkA*-expressing tumors showed many classical characteristics of primary ganglioneuroblastoma, demonstrating the reversion of a highly aggressive tumor into a differentiated malignancy by a *trkA*-mediated signal cascade.

FIG. 6. Northern blot analysis of marker genes of neuronal differentiation. Total RNA was isolated at the indicated time intervals and hybridized with probes for the SCG-10 gene (A) or the *ret* proto-oncogene (B). A low level of SCG-10 transcript is present in untreated transfectants; however, a strong activation occurs after long-term NGF treatment. The blot was rehybridized with a probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The *ret* proto-oncogene was not transcribed at a detectable level in untreated transfectants. Strong *ret* gene activation results in multiple-molecular-weight transcripts in NGF-treated cells. The blot was rehybridized with a probe for GAPDH to test for RNA loading.

FIG. 7. Histopathology and immunohistological analysis of nude mouse tumors. Untreated (A, C, and E) and NGF-treated (B, D, and F) tumors were stained with either hematoxylin and eosin or anti-S100 antibodies counterstained with hematoxylin only (see Materials and Methods). Hematoxylin-eosin staining (A and B) demonstrated the dramatic change in the histopathology following NGF treatment. The treated tumor tissue showed many characteristics of ganglioneuroblastoma, including a vast amount of stroma and mature neurons (arrowheads). (Insert) High-power magnification of a phenotypically differentiated neuron following NGF treatment. (Magnification, $\times 498$.) Staining with anti-S100 α (C and D) and anti-S100 β (E and F) antibodies of NGF-treated tumor tissue demonstrated a strong immunoreactivity for the β subunit and less for the α subunit. Untreated tumors showed only background staining with both antibodies. (Magnification, $\times 83$.)

DISCUSSION

We have investigated the role of *trkA* in NB cell growth and differentiation in vitro and in vivo. A malignant NB cell line, which lacks expression of both the endogenous *trkA* and the *gp75^{NGFR}* genes, has been stably transfected with the human *trkA* cDNA. Transfectants express surface-bound *trkA* receptors which when activated with NGF mediate a cascade of cellular signals leading to terminal differentiation of NB cells in vitro. We further demonstrate bimodal differentiation of subcutaneous tumors derived from *trkA*-transfected NB cells following injection of NGF. Therefore, *trkA* mediates an NGF signal in the absence of *gp75^{NGFR}* molecules that reverses a malignant NB into a differentiated tumor cell in vitro and in vivo.

These observations raise many important questions relevant to the biology of neurotrophins and their receptors in human neural crest cell-derived tumors. First, how many receptors are needed in NB cells to generate a functional signal following NGF treatment? Various *trkA*-transfected HTLA230 clones showed different levels of *trkA* mRNA. Activation with NGF of the receptor signal pathway induced *c-fos* gene transcription in all clones analyzed independent of the level of *trkA* message. FACS analysis of *trkA* transfectants showed heterogeneous *trkA* surface expression varying from high to background fluorescence. Yet more than 90% of transfectants, when treated with NGF in vitro, showed morphological changes. Thus, NB cells with few *trkA* molecules are able to functionally respond, and FACS analysis was not sensitive enough to discriminate between low-level *trkA* expression and background fluorescence. This observation is consistent with other data demonstrating that human NB cell lines with few high-affinity binding sites for NGF respond to NGF (2, 35, 53). Although we attempted to determine the number of NGF-binding sites in *trkA* transfectants, it became evident that such a number would be meaningless given the broad anti-*trkA* fluorescence histogram. Transfection of genes encoding surface markers has been shown to generate heterogeneous cell populations despite repetitive subcloning (37). Such heterogeneity may be due to various cellular properties and could further be enhanced by the instability of the cell line. Nevertheless, the number of *trkA* receptors per NB cell required for a functional signal may be low and possibly similar to that seen in PC12 cells (23). This observation may bear important implications for primary NB in patients. We and others have found that most primary differentiated ganglioneuroblastomas express various levels of *trkA* mRNA, although little is known about the number, cellular distribution, and function of *trkA* molecules in these tumors (41, 55).

Second, what is the binding affinity for NGF of the transfected *trkA*? Transfection of the *trkA* cDNA into non-neuronal cells resulted in intermediate- to high-affinity binding sites in addition to a majority of low-affinity binding sites (27). Expression in mutated PC12 cells with low *gp75^{NGFR}* gene expression generated high-affinity NGF binding sites (33). Similar results were observed in 18-10 cells by using a scintillation proximity assay (23) (data not shown). Thus, the exact cellular requirements of *trkA* to generate high-affinity binding sites for NGF remain elusive.

Third, is *gp75^{NGFR}* expression required for an NGF-mediated signal in NB cells? We and others have previously observed that the transfected *gp75^{NGFR}* was able to signal an NGF response in several cell lines, although the molecular mechanisms have not been identified (16, 37, 43). In the present study, we demonstrate that the transfected *trkA*

gene reconstituted a membrane receptor that was capable of signalling a cellular response in NB cells, leading to growth arrest and differentiation in the absence of *gp75^{NGFR}*. However, one cannot rule out that *trkA* expression by gene transfer creates an artificial intracellular environment that allows NB cells to respond to NGF in the absence of *gp75^{NGFR}* molecules. It is worth mentioning that *trkA* and its related family members induce cell growth and transformation rather than differentiation in the presence of the preferred ligand in nonneuronal cells. Thus, additional neuronal cell-specific components that distinguish a differentiation from a mitogenic response independent of *gp75^{NGFR}* are required and present in HTLA230 cells. It is worth mentioning that no message for either the *trkB* or the *trkC* gene could be found in mock-transfected HTLA230-neo cells, and treatment with either BDNF or NT3 did not activate immediate-early genes (data not shown).

The *N-myc* oncogene was significantly down-regulated following NGF treatment, and only a residual level of *N-myc* gene transcription was present as a result of the presence of NGF-nonresponsive cells. This finding was substantiated by autoradiographic analysis of DNA synthesis in NGF-treated cells. The *N-myc* gene is expressed during embryogenesis in proliferative neuroblastic precursor cells and is silent in postmitotic mature neurons (26). Most aggressive NB and cultured NB cell lines, including HTLA230, have high *N-myc* gene amplification and *N-myc* gene overexpression, indicative of a poor prognosis. In contrast, ganglioneuroblastomas, which generally have a good prognosis, do not show *N-myc* gene amplification and have only low *N-myc* gene expression (50). The *trkA*-mediated NGF signal pathway transcriptionally inactivated the *N-myc* oncogene in transfectants, although the gene shows a 20-fold amplification. This is the first demonstration that a neurotrophic factor-mediated cascade regulates a cell cycle-controlling *myc* gene. Inactivation of the *N-myc* gene enables postmitotic NB cells to activate various differentiation-specific genes. In contrast, HTLA230 cells, which were morphologically differentiated in the presence of retinoic acid, showed an unchanged level of *N-myc* gene transcription, and no differentiation-specific genes were activated (39). Thus, inactivation of the *N-myc* gene by the NGF signal cascade generates growth-arrested tumor cells with a differentiated phenotype.

The in vitro experiments were performed with transfectants grown on the R₂₂C1F matrix. The ECM has long been shown to mediate signals effecting cell growth, differentiation, and gene expression (1, 22). We have recently demonstrated that the R₂₂C1F matrix promotes differentiation of neuronal cells and that the composition of the glycosaminoglycans in the R₂₂C1F matrix is a determining factor for NB cell differentiation in vitro (38). The ECM has also been shown to bind cellular factors such as basic fibroblast growth factor (bFGF), which binds to glycosaminoglycan components (46). The growth factor induces phenotypic changes in some neuronal precursor cells. We do not yet know whether bFGF is present in the R₂₂C1F matrix; however, addition of purified bFGF to either HTLA230-neo cells or 18-10 transfectants does not promote differentiation. It is conceivable that the R₂₂C1F matrix enhanced NGF-induced neuronal differentiation in *trkA* transfectants, although immediate-early gene activation and phenotypic changes were also observed in NGF-treated transfectants grown on plastic or gelatin-coated dishes.

Bimodal differentiation of cultured NB cell lines by using retinoic acid has been demonstrated (39). Treatment of tumor-bearing mice with NGF for 6 consecutive days in-

duced differentiation along both the Schwannian and neuronal pathway in tumors of transfectants but not parental cells. In contrast, *trkA* transfectants treated in vitro with NGF showed differentiation along the neuronal pathway only. This discrepancy could be explained in that NGF-responsive NB synthesize factors promoting Schwann cell maturation which may not have the necessary biological activities under tissue culture conditions. Alternatively, Schwann cell differentiation in vivo may also require host cell factors, absent in tissue culture. Nevertheless, the NGF signal cascade induced differentiation along the neuronal and glial pathway in NB, an observation that so far has not been described.

Schwann cells are a major component of differentiated ganglioneuroblastomas. Differentiation induction in *trkA*-expressing tumors by NGF demonstrates that aggressive NB can be phenotypically reversed in vivo by NGF, hence opening novel treatment modalities for patients with NB. Reversion of an aggressive NB with *N-myc* gene amplification also suggests that an aggressive NB may develop from a ganglioneuroblastoma by tumor progression. Loss of a functional NGF signal cascade may be one factor contributing to NB tumor progression. This observation is supported by our findings that most aggressive NB do not express the endogenous *trkA* gene and that all stage IVS tumors analyzed showed high *trkA* expression (55). These latter tumors have been described to spontaneously differentiate and regress. The established *trkA* transfectants support the hypothesis that spontaneous tumor regression could be the result of an activated NGF signal cascade in some NB.

In conclusion, we demonstrate that NGF mediates a differentiation response in aggressive NB expressing *trkA*. *gp75^{NGFR}* is not required for the NGF signal pathway in NB. Furthermore, NGF and other neurotrophic factors may reverse tumor progression in responsive cells similarly to what is seen with stage IVS tumors.

ACKNOWLEDGMENTS

We thank M. Barbacid, K. Ikenaka, R. Kuwano, N. Nomura, M. Nagao, A. Kikuchi, M. Fishman, B. Shafit-Zagardo, and D. Leonard for cDNA clones. Furthermore, we acknowledge K. Eager and K. Hayashi for providing antibodies. We thank K. Maekawa and Y. Eto for generous support, and we thank J. Blanchard, who typed the manuscript.

This study was supported by a grant from the National Center for Nervous, Mental, and Muscular Disorders of the Ministry of Health and Welfare and a grant-in-aid for scientific research from the Ministry of Education, Japan (H. Matsushima) as well as NINDS grants NS25795 and EY04950 from the National Institutes of Health and the T. J. Martell Foundation (E. Bogenmann).

REFERENCES

- Adams, J. C., and F. M. Watt. 1989. Fibronectin inhibits the terminal differentiation of human keratinocytes. *Nature (London)* **340**:307-309.
- Azar, C. G., N. J. Scavarda, C. P. Reynolds, and G. M. Brodeur. 1990. Multiple defects of the nerve growth factor receptor in human neuroblastomas. *Cell Growth Differ.* **1**:421-428.
- Birren, S. J., and D. J. Anderson. 1990. A v-myc-immortalized sympathoadrenal progenitor cell line in which neuronal differentiation is initiated by FGF but not NGF. *Neuron* **4**:189-201.
- Bothwell, M. 1991. Tissue localization of nerve growth factor and nerve growth factor receptors. *Curr. Top. Microbiol. Immunol.* **165**:55-70.
- Chao, M. V. 1992. Neurotrophin receptors: a window into neuronal differentiation. *Neuron* **9**:583-593.
- Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294-5299.
- Cowan, N. J., P. R. Dobner, E. V. Fuchs, and D. W. Cleveland. 1983. Expression of human alpha-tubulin genes: interspecies conservation of 3' untranslated regions. *Mol. Cell. Biol.* **3**:1738-1745.
- Curran, T., G. Peters, C. Van Beveren, N. M. Teich, and I. M. Verma. 1982. FBJ murine osteosarcoma virus: identification and molecular cloning of biologically active proviral DNA. *J. Virol.* **44**:674-682.
- Dammerman, M., S. H. Yen, and B. Shafit Zagardo. 1989. Sequence of a human MAP-2 region sharing epitopes with Alzheimer neurofibrillary tangles. *J. Neurosci. Res.* **24**:487-495.
- Eager, K. B. 1991. Molecular characterization of human trk proto-oncogene product monoclonal antibodies. *Oncogene* **6**:819-824.
- Giard, D. J., S. A. Aaronson, G. J. Todaro, P. Arnstein, J. H. Kersey, H. Dosik, and W. P. Parks. 1973. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J. Natl. Cancer Inst.* **51**:1417-1423.
- Grady Leopardi, E. F., M. Schwab, A. R. Ablin, and W. Rosenau. 1986. Detection of N-myc oncogene expression in human neuroblastoma by in situ hybridization and blot analysis: relationship to clinical outcome. *Cancer Res.* **46**:3196-3199.
- Greenberg, M. E., L. A. Greene, and E. B. Ziff. 1985. Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. *J. Biol. Chem.* **260**:14101-14110.
- Hattori, K., P. Angel, M. M. Le Beau, and M. Karin. 1988. Structure and chromosomal localization of the functional intronless human JUN protooncogene. *Proc. Natl. Acad. Sci. USA* **85**:9148-9152.
- Hempstead, B. L., D. Martin Zanca, D. R. Kaplan, L. F. Parada, and M. V. Chao. 1991. High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. *Nature (London)* **350**:678-683.
- Hempstead, B. L., L. S. Schleifer, and M. V. Chao. 1989. Expression of functional nerve growth factor receptors after gene transfer. *Science* **243**:373-375.
- Ibanez, C. F., T. Ebendal, G. Barbany, J. Murray Rust, T. L. Blundell, and H. Persson. 1992. Disruption of the low affinity receptor-binding site in NGF allows neuronal survival and differentiation by binding to the trk gene product. *Cell* **69**:329-341.
- Ignatius, M. J., C. R. Chandler, and E. M. Shooter. 1985. Nerve growth factor-treated, neurite-bearing PC12 cells continue to synthesize DNA. *J. Neurosci.* **5**:343-351.
- Ikenaka, K., K. Nakahira, C. Takayama, K. Wada, H. Hatanaka, and K. Mikoshiba. 1990. Nerve growth factor rapidly induces expression of the 68-kDa neurofilament gene by post-transcriptional modification in PC12h-R cells. *J. Biol. Chem.* **265**:19782-19785.
- Ip, N. Y., T. N. Stitt, P. Tapley, R. Klein, D. J. Glass, J. Fandl, L. A. Greene, M. Barbacid, and G. D. Yancopoulos. 1993. Similarities and differences in the way neurotrophins interact with the Trk receptors in neuronal and nonneuronal cells. *Neuron* **10**:137-149.
- Ito, E., N. Nomura, and R. Narayanan. 1990. Transcriptional regulation of early growth response genes in FOS-expressing PC-12 cells. *Cell Regul.* **1**:347-357.
- Jessell, T. M. 1988. Adhesion molecules and the hierarchy of neural development. *Neuron* **1**:3-13.
- Jing, S., P. Tapley, and M. Barbacid. 1992. Nerve growth factor mediates signal transduction through trk homodimer receptors. *Neuron* **9**:1067-1079.
- Johnson, D., A. Lanahan, C. R. Buck, A. Sehgal, C. Morgan, E. Mercer, M. Bothwell, and M. Chao. 1986. Expression and structure of the human NGF receptor. *Cell* **47**:545-554.
- Karns, L. R., S. C. Ng, J. A. Freeman, and M. C. Fishman. 1987. Cloning of complementary DNA for GAP-43, a neuronal growth-related protein. *Science* **236**:597-600.
- Kato, K., A. Kanamori, Y. Wakamatsu, S. Sawai, and H. Kondoh. 1991. Tissue distribution of N-myc expression in the

- early organogenesis period of the mouse embryo. *Dev. Growth Differ.* 33:29–36.
27. Klein, R., S. Q. Jing, V. Nanduri, E. O. Rourke, and M. Barbacid. 1991. The *trk* proto-oncogene encodes a receptor for nerve growth factor. *Cell* 65:189–197.
 28. Kuwano, R., H. Usui, T. Maeda, K. Araki, T. Yamakuni, T. Kurihara, and Y. Takahashi. 1987. Tissue distribution of rat S-100 alpha and beta subunit mRNAs. *Brain Res.* 388:79–82.
 29. Kuwano, R., H. Usui, T. Maeda, T. Fukui, N. Yamanari, E. Ohtsuka, M. Ikehara, and Y. Takahashi. 1984. Molecular cloning and the complete nucleotide sequence of cDNA to mRNA for S-100 protein of rat brain. *Nucleic Acids Res.* 12:7455–7465.
 30. Lee, K. F., E. Li, L. J. Huber, S. C. Landis, A. H. Sharpe, M. V. Chao, and R. Jaenisch. 1992. Targeted mutation of the gene encoding the low affinity NGF receptor *p75* leads to deficits in the peripheral sensory nervous system. *Cell* 69:737–749.
 31. Leonard, D. G., E. B. Ziff, and L. A. Greene. 1987. Identification and characterization of mRNAs regulated by nerve growth factor in PC12 cells. *Mol. Cell. Biol.* 7:3156–3167.
 32. Loeb, D. M., and L. A. Greene. 1993. Transfection with *trk* restores “slow” NGF binding, efficient NGF uptake, and multiple NGF responses to NGF-nonresponsive PC12 cell mutants. *J. Neurosci.* 13:2919–2929.
 33. Loeb, D. M., J. Maragos, D. Martin Zanca, M. V. Chao, L. F. Parada, and L. A. Greene. 1991. The *trk* proto-oncogene rescues NGF responsiveness in mutant NGF-nonresponsive PC12 cell lines. *Cell* 66:961–966.
 34. MacLeod, A. R. 1981. Expression of the mRNA coding for glyceraldehyde-3-phosphate dehydrogenase. *Eur. J. Biochem.* 119:353–358.
 35. Marchetti, D., and J. R. Perez Polo. 1987. Nerve growth factor receptors in human neuroblastoma cells. *J. Neurochem.* 49:475–486.
 36. Martin Zanca, D., R. Oskam, G. Mitra, T. Copeland, and M. Barbacid. 1989. Molecular and biochemical characterization of the human *trk* proto-oncogene. *Mol. Cell. Biol.* 9:24–33.
 37. Matsushima, H., and E. Bogenmann. 1990. Nerve growth factor (NGF) induces neuronal differentiation in neuroblastoma cells transfected with the NGF receptor cDNA. *Mol. Cell. Biol.* 10:5015–5020.
 38. Matsushima, H., and E. Bogenmann. 1992. Modulation of neuroblastoma cell differentiation by the extracellular matrix. *Int. J. Cancer* 51:727–732.
 39. Matsushima, H., and E. Bogenmann. 1992. Bi-modal differentiation pattern in a new human neuroblastoma cell line in vitro. *Int. J. Cancer* 51:250–258.
 40. Meakin, S. O., and E. M. Shooter. 1991. Molecular investigations on the high-affinity nerve growth factor receptor. *Neuron* 6:153–163.
 41. Nakagawara, A., M. Arima Nakagawara, N. J. Scavarda, C. G. Azar, A. B. Cantor, and G. M. Brodeur. 1993. Association between high levels of expression of the *TRK* gene and favorable outcome in human neuroblastoma. *N. Engl. J. Med.* 328:847–854.
 42. Nomura, N., M. Ide, S. Sasamoto, M. Matsui, T. Date, and R. Ishizaki. 1990. Isolation of human cDNA clones of *jun*-related genes, *jun-B* and *jun-D*. *Nucleic Acids Res.* 18:3047–3048.
 43. Pleasure, S. J., U. R. Reddy, G. Venkatakrishnan, A. K. Roy, J. Chen, A. H. Ross, J. Q. Trojanowski, D. E. Pleasure, and V. M. Lee. 1990. Introduction of nerve growth factor (NGF) receptors into a medulloblastoma cell line results in expression of high- and low-affinity NGF receptors but not NGF-mediated differentiation. *Proc. Natl. Acad. Sci. USA* 87:8496–8500.
 44. Radeke, M. J., T. P. Misko, C. Hsu, L. A. Herzenberg, and E. M. Shooter. 1987. Gene transfer and molecular cloning of the rat nerve growth factor receptor. *Nature (London)* 325:593–597.
 45. Rodriguez Tebar, A., G. Dechant, R. Goetz, and Y. A. Barde. 1992. Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. *EMBO J.* 11:917–922.
 46. Ruoslahti, E., and Y. Yamaguchi. 1991. Proteoglycans as modulators of growth factor activities. *Cell* 64:867–869.
 47. Sano, K., H. Nakamura, S. Mabuchi, T. Tanaka, A. Nakagawara, and Y. Takai. 1990. Expression of the *smg p25A* (a *ras* p21-like GTP-binding protein) gene in human neuroblastoma cell lines and tumor tissues. *Cancer Res.* 50:7242–7245.
 48. Schlessinger, J., and A. Ullrich. 1992. Growth factor signaling by receptor tyrosine kinases. *Neuron* 9:383–391.
 49. Schwab, M., K. Alitalo, K. H. Klempner, H. E. Varmus, J. M. Bishop, F. Gilbert, G. Brodeur, M. Goldstein, and J. Trent. 1983. Amplified DNA with limited homology to *myc* cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature (London)* 305:245–248.
 50. Seeger, R. C., G. M. Brodeur, H. Sather, A. Dalton, S. E. Siegel, K. Y. Wong, and D. Hammond. 1985. Association of multiple copies of the *N-myc* oncogene with rapid progression of neuroblastomas. *N. Engl. J. Med.* 313:1111–1116.
 51. Seeger, R. C., S. E. Siegel, and N. Sidell. 1982. Neuroblastoma: clinical perspectives, monoclonal antibodies, and retinoic acid. *Ann. Intern. Med.* 97:873–884.
 52. Sheng, M., and M. E. Greenberg. 1990. The regulation and function of *c-fos* and other immediate early genes in the nervous system. *Neuron* 4:477–485.
 53. Sonnenfeld, K. H., and D. N. Ishii. 1985. Fast and slow nerve growth factor binding sites in human neuroblastoma and rat pheochromocytoma cell lines: relationship of sites to each other and to neurite formation. *J. Neurosci.* 5:1717–1728.
 54. Stein, R., S. Orit, and D. J. Anderson. 1988. The induction of a neural-specific gene, *SCG10*, by nerve growth factor in PC12 cells is transcriptional, protein synthesis dependent, and glucocorticoid inhibitable. *Dev. Biol.* 127:316–325.
 55. Suzuki, T., E. Bogenmann, H. Shimada, D. Stram, and R. C. Seeger. 1993. Lack of high-affinity nerve growth factor receptors in aggressive neuroblastomas. *J. Natl. Cancer Inst.* 85:377–384.
 56. Tahira, T., Y. Ishizaka, F. Itoh, M. Nakayasu, T. Sugimura, and M. Nagao. 1991. Expression of the *ret* proto-oncogene in human neuroblastoma cell lines and its increase during neuronal differentiation induced by retinoic acid. *Oncogene* 6:2333–2338.
 57. Takahashi, K., T. Isobe, Y. Ohtsuki, T. Akagi, H. Sonobe, and T. Okuyama. 1984. Immunohistochemical study on the distribution of alpha and beta subunits of S-100 protein in human neoplasm and normal tissues. *Virchows Arch. B* 45:385–396.
 58. Tapley, P., F. Lamballe, and M. Barbacid. 1992. K252a is a selective inhibitor of the tyrosine protein kinase activity of the *trk* family of oncogenes and neurotrophin receptors. *Oncogene* 7:371–381.
 59. Torres, M., F. L. Hall, and K. O. Neill. 1993. Stimulation of human neutrophils with formyl-methionyl-leucyl-phenylalanine induces tyrosine phosphorylation and activation of two distinct mitogen-activated protein-kinases. *J. Immunol.* 150:1563–1577.
 60. Weskamp, G., and L. F. Reichardt. 1991. Evidence that biological activity of NGF is mediated through a novel subclass of high affinity receptors. *Neuron* 6:649–663.
 61. Yaeger, M. J., A. Koestner, K. Marushige, and Y. Marushige. 1992. The use of nerve growth factor as a reverse transforming agent for the treatment of neurogenic tumors: in vivo results. *Acta Neuropathol.* 83:624–629.