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Laboratory Investigation

Differentiation and growth inhibition of glioma cells induced by transfer of *trk* A proto-oncogene

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Key words: glioma, differentiation, nerve growth factor (NGF), NGF receptor, *trk* A proto-oncogene

Summary

The induction of growth inhibition and differentiation of a glioma cell line by transfection of *trk* A cDNA was examined, and production of endogenous nerve growth factor (NGF) also was studied in these cells. When human *trk* A cDNA was transfected into a human glioma cell line, U-251MG, which lacks expression of both endogenous *trk* A and low-affinity NGF receptor, the transfectant expressed the exogenous *trk* A mRNA and a functional high-affinity NGF receptor. Transfection of *trk* A cDNA caused a partial induction of cell differentiation, G1 arrest, growth inhibition, tyrosine phosphorylation of the *trk* A proto-oncogene product, and activation of MAP kinase. Exogenous NGF treatment induced further terminal differentiation and growth inhibition. In summary, our data suggest that endogenous NGF secreted by glioma cells has an important role in the induction of glioma-cell differentiation occuring with transfer of exogenous *trk* A cDNA.

Introduction

Malignant gliomas are the most common primary intracranial malignant tumor, accounting for 40% of primary brain tumors in many series [1]. In spite of aggressive surgical therapy, radiotherapy, and chemotherapy of patients with these tumors, the overall 5-year survival is $<5.5\%$ [2]. These abysmal survival rates demand a search for new modalities of therapy.

Gene transfer is a strategy in which the genetic control of neoplastic cells is modified for a therapeutic purpose or to help gain understanding of the biology of neoplastic cells. Two distinct clinical strategies for human gene therapy have been used in the treatment of neoplastic disease. One is gene replacement or excision and the other is gene addition. In gene addition therapy directed at human neoplastic cells, one theoretically could deliver a defined sequence of a normal gene relating to differentiation to these cells [3].

Nerve growth factor (NGF) has been extensively characterized as a survival factor for neurons [4,5]. Two structurally unrelated NGF receptors have been identified. The low-affinity NGF receptor, with a dissociation constant (Kd) of 10^{-9} M, is a single transmembrane glycoprotein with no identifiable catalytic activity in its cytoplasmic domain [6,7]. In contrast, the NGF tyrosine kinase receptor, tropomyosine receptor kinase (trk) A displays a higher affinity, with Kd of 10^{-11} M [8,9]. High-affinity binding is necessary to mediate the biologic action of NGF [10,11]. In neuroblastoma, *trk* A gene expression is associated with lower disease stage and favorable outcome, and the absence of *trk* A mRNA expression is associated with tumor progression [12]. Transfection of *trk* A complementary (c) DNA into a human neuroblastoma cell line lacking expression of both endogenous *trk* A proto-oncogene product and low-affinity NGF receptor caused enhanced growth arrest and differentiation in the presence of NGF [13–15]. Similar transfer has not been examined in glioma cells.

The present study demonstrates that transfection of human *trk* A cDNA into human malignant glioma cells reconstitutes a functional NGF-mediated signal cascade that leads to growth arrest and differentiation.

We further demonstrate that NGF of tumor-cell origin brings about autocrine induction of differentiation in human glioma cells transfected with *trk* A cDNA.

Materials and methods

Cloning of human trk *A proto-oncogene and preparation of expression plasmids*

Poly (A)+ RNA obtained from a human medulloblastoma cell line, MED-H, which showed high expression of the *trk* A proto-oncogene product [16], was used to make an oligo (dT)-primed cDNA library with the SuperScript Lambda System (GIBCO, Grand Island, NY). A total 100 ng of cDNA, comprising the 1.5–12 kb fractions, was ligated to λgt22A Not 1-Sal 1 arms (GIBCO), and phage clones were obtained following the injection of *E. coli* strain Y1090(r^-). Phage colonies were screened by hybridization of colony lifts to *trk* A cDNA probe prepared as described below. The cDNA inserts isolated from the libraries were subcloned into the pBluescript II plasmid vector (Stratagene, San Diego, CA) and subjected to cycle sequencing using ΔTth DNA polymerase (Sequencing PRO autosequencer core kit for labeled primer, TOYOBO, Tokyo, Japan). The sequences were determined using primers for Bluescript II phagemid vectors (TOYOBO) and analyzed with the Shimazu autosequencer model DSQ-1 (Shimazu, Tokyo, Japan). A homology search program, FASTA was performed in the GenBank and EMBL nucleic acid databases using an on-line system through DNA Data Bank of Japan (Mishima, Japan). The nucleotide sequence of part of this fragment was identical to a previously reported human *trk* A cDNA [17]. A *trk* A cDNA clone (*trk* 7) containing the 2,300-bp sequence was subcloned into the Hind III site of expression vector pRc/CMV (Invitrogen, San Diego, CA) with a Hind III linker. The human *trk* A cDNA probe was prepared by the PCR using template cDNA in MED-H cells. The primer pairs used were 5-CCATCGTGAAGAGTGGTCCTC-3′ and 5′-GGTGACATTGGCCAGGGTCA-3′. The expected size of the PCR product was 476 bp. The PCR reaction was performed in 100 μl of a reaction mixture containing 20 ng of template cDNA, a pair of primers, 60 μM of each deoxynucleotide triphosphate, 10 mM Tris-HCl buffer (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl and 2.5 units of Takara LA Taq (TAKARA SHUZO, Shiga, Japan). To amplify the *trk* A gene, 30 cycles of denaturation (94°C, 0.5 min), annealing (60°C, 0.5 min) and extension (72°C, 1.5 min) were performed in a thermal cycler (ZYMOREACTOR II, AB-1820, ATTO, Tokyo, Japan).

Cells and gene transfer

The human medulloblastoma cell line MED-H and the glioma cell line U-251MG were maintained in RPMI-1640 medium (GIBCO) supplemented with 10% fetal calf serum and kanamycin sulfate (50 μg/ml), and grown in monolayer culture at 37°C. The MED-H cell line was developed in our laboratory from a cerebellar medulloblastoma [16], and the U-251MG cell line was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan).

The expression vector pRc/CMV/*trk* contains a 2,300 bp human *trk* A cDNA, a CMV promoter, a neomycin-resistance gene, and an SV40 early promoter signal. For transfection, semiconfluent U-251MG cells were cultured in serum-free medium, and 20 μg of plasmid DNA and 30 μg of lipofectin reagent (Bethesda Research Laboratories Life Technologies, Bethesda, MD) were added. Transfected cells were incubated overnight in non-selective medium and then cultured in growth medium containing 500 μg/ml of G418 (Sigma Chemical, St. Louis, MO) at 72 h post-transfection. G418-resistant colonies were collected and then plated at limiting dilution in 96-well tissue culture plates. Selected transfectants were maintained in medium as above with G418 at 500 μg/ml. Transfectant with pRc/CMV plasmid only was used as a negative control.

Expression of trk *A proto-oncogene mRNA*

RNA was isolated from cells by homogenization in guanidium thiocyanate solution followed by cesium chloride centrifugation [18]. Total RNAs were separated electrophoretically on 2.2 M formaldehyde/agarose gels, blotted onto nitrocellulose filters, and then hybridized in 5× SSC/50% formamide for 16 h at 42°C with human *trk* A cDNA probe labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (TAKARA SHUZO). Labeling of probe obtained from PCR was done in a solution containing 50% formamide, 10% dextran sulfate, 1 M NaCl, 1% sodium dodecyl sulfate (SDS), 1× Denhardt's solution, 25 mM Tris-HCI (pH 7.4) and 50 μg/ml denatured salmon sperm DNA. Washes used 2× SSC and 0.1% SDS at room temperature, followed by 68°C.

Expression of trk *A proto-oncogene product analyzed with immunoblot assay*

Cells were disrupted with 500 μl of cell lysing buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.01% SDS, 0.15 M NaCl, 50 mM Tris-HCl (pH 7.4) and 2 mM phenylmethyl sulfonylfluoride. Lyophilized samples were dissolved at 1 mg/ml of protein concentration in a buffer containing 2.5% SDS, 5% 2-mercaptoethanol and 10 mM Tris-HCl (pH 8.0), and boiled for 5 min at 100°C. SDS-PAGE was performed following the method of Laemmli [19]. Electrophoresis was performed at room temperature for 4 h at 20 mA on a 0.4 mm thick, 10% gel plate. Proteins were transferred electrophoretically onto nitrocellulose filters at a constant voltage of 10 V for 4 h as described by Towbin et al. [20]. The filters were blocked with 5% non-fat milk then anti-*trk* A antibody (No. 763, Santa Cruz Biotechnology, Santa Cruz, CA) was used for immunoblotting. Signals were detected using an Amersham ECL system (Amersham Japan, Tokyo, Japan).

Analysis of ^{125}*I-NGF binding*

2.5 S NGF (Biomedical Technologies, Stoughton, MA) was iodinated by the lactoperoxidase method of Sutter et al. [21] (specific activity, 1×10^8 cpm/μg). Binding of ^{125}I-NGF to cells was assayed according to the method by Vale and Shooter [22]. Nonspecific binding of ^{125}I-NGF was determined in the presence of 500 nM NGF and was subtracted from all data. Nonspecific binding was under 10% of total binding.

Analysis of GFAP expression

Cellular expression of glial fibrillary acidic protein (GFAP) used as a marker of astrocytic differentiation was assayed by immunoblotting with anti-GFAP antibody (clone: 6F-2, DAKO Japan, Kyoto, Japan). Anti-β actin antibody was used as an internal control to estimate general level of protein synthesis.

Population doubling time and transplantability in SCID mice

Growth curves for cells were obtained by seeding them in 25 cm^2 plastic culture dishes at a concentration of 3×10^4 cells in 3 ml of medium. After the incubation period, viable cells were counted by the trypan-blue exclusion method and population doubling times were calculated. Experiments were done in triplicate and statistical analysis was done using Student's *t* test.

Tumorigenicity of cell lines was tested by sc injection of 5×10^6 cells suspended in 0.2 ml of growth medium into 6-week-old C.B-17 severe combined immunodeficiency (SCID) mice purchased from Clea Japan, Tokyo, Japan.

Analysis of tyrosine phosphorylation of trk *A proto-oncogene product and MAP kinase*

For immunoprecipitation, cells with or without NGF treatment were washed with cold phosphate-buffered saline, pH 7.4 (PBS) and dissolved on ice in lysing buffer containing 1% NP-40, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethyl sulfonylfluoride, aprotinine (0.15 U/ml), 20 μM leupeptin and 1 mM sodium vanadate, and rocked gently for 20 min at 4°C. Insoluble material was removed by centrifugation at 4°C for 10 min at 10,000× *g*. Cell lysates were normalized for protein content before immunoprecipitation assays. Immunoprecipitations were performed by incubating lysates with anti-*trk* A antibody or anti-mitogen-activated protein (MAP) kinase (Santa Cruz Biotechnology) for 6 h at 4°C. Protein G-sepharose was used to collect the antigen–antibody complexes. Immunoprecipitates then were washed three times with cold lysing buffer and once with 10 mM Tris-HCl buffer (pH 7.4) before analysis. SDS-PAGE and immunoblotting were performed following previously outlined methods. Filters were blocked with 5% non-fat milk, and then anti-phosphotyrosine monoclonal antibody (clone: PY 20, ICN Biochemicals, Costa Mesa, CA) was used for immunoblotting. Antibody binding to filters was detected by the Amersham ECL system (Amersham Japan).

*Analysis of expression of c-*fos *mRNA*

The expression of c-*fos* mRNA was analyzed using the method described for expression of *trk* A proto-oncogene mRNA with the c-*fos* cDNA fragment being cut with EcoR1 from a pSPT-*fos* cDNA plasmid (Japanese Cancer Research Resources Bank, Tokyo, Japan).

Cell-cycle analysis

Cell-cycle analysis was performed as previously described [23]. Cells in the exponentially growing

phase were labeled with 10 μM bromodeoxyuridine (BrdU, Sigma) and incubated at 36°C for 2 h. Cells were washed twice with PBS, trypsinized, centrifuged and washed twice with PBS/1% bovine serum albumin (BSA). Final cell pellets were fixed with 70% ethanol for 30 min on ice. Before staining for BrdU with anti-BrdU antibody conjugated to fluorescein isothiocyanate (FITC, Becton Dickinson, Mountain View, CA), the cell pellet was denatured in 2 N HCl/Triton X-100 for 30 min, then neutralized in 0.1 M $Na_2B_4O_7$, pH 8.5. Cells (10^6) suspended in 0.5% Tween 20/PBS/1% BSA solution were used for the antibody reaction. Before analysis, cell pellets were suspended in PBS containing 5 μg/ml propidium iodide. Cells (10^5) were analyzed using a fluorescence-activated cell sorter (FACScan, Becton-Dickinson) for both DNA content and BrdU incorporation.

Analysis of NGF mRNA using RT-PCR

Total RNA was prepared from cells by the guanidium thiocyanate procedure, and converted to single-stranded cDNA by reverse transcriptase with oligo (dT) primer. Synthesized cDNA was used in PCR as a template with two sets of oligonucleotide primers. The primer pairs used were 5′-CCAAGGACGCAGCTTTCTAT-3′ and 5′-CTCCGGTGAGTCCTGTTGAA-3′ and the expected size of the PCR product was 735 bp. PCR was carried out in 30 temperature cycles consisting of 94°C, 55°C, and 72°C for periods of 1, 2, and 3 min, respectively in a thermal cycler. The PCR product using the primers of G3PDH was an internal control.

Analysis of NGF expression and NGF production in culture media

Expression of cellular NGF was analyzed using immunoblot assays with anti-NGF antibody (No. # sc-548, Santa Cruze Biotechnology). NGF protein levels in cultured media were measured by two-site enzyme immunoassay [24] using anti-β-NGF and β-galactosidase labeled anti-β-NGF (clone: 27/21, Boehringer Mannheim, Tokyo, Japan). Experiments determining production of NGF in culture media were done in triplicate, and statistical analysis was performed using Student's *t* test.

Results

Characterization of transfectant introduced with human trk *A cDNA*

U-251MG cells were transfected with human *trk* A expression vector. Six clonal cell lines of G418-resistant transfectants were obtained, and one clone with high expression of *trk* A proto-oncogene (U-251MG/*trk*) was used in these experiments. Although U-251MG cells did not express the endogenous *trk* A proto-oncogene and its product (Figure 1A and B), U-251MG/*trk* transfected with human *trk* A cDNA showed the expression of exogenous *trk* A proto-oncogene by Northern blot and its product by Western blot. U-251MG/pRc transfected with control vector without *trk* A cDNA did not express *trk* A proto-oncogene or its product (Figure 1A and B). U-251MG cells did not contain low-affinity NGF receptor (data not shown). U-251MG/*trk* cells were tested for specific NGF binding compared with PC-12 cells with low- and high-affinity NGF binding. As shown in Table 1, PC-12 cells contained low- and high-affinity NGF binding sites, but U-251MG/*trk* cells showed only the relatively high-affinity NGF binding sites. The dissociation constant (Kd) of U-251MG/*trk* cells was 0.72×10^{-11} M, and number of binding sites/cell was 2.8×10^4, a greater receptor density than that of high-affinity binding sites of PC-12 cells. U-251MG and U-251MG/pRc did not show any NGF binding.

Population doubling times and transplantability into SCID mice (Table 2)

The population doubling time of U-251MG cells was 36.1 ± 3.68 h and did not differ from that of U-251MG/pRc. The population doubling time of U-251MG/*trk* cells was 52.1 ± 4.21 h, which was significantly longer than that of U-251MG cells ($P < 0.05$). Furthermore, the treatment with 20 ng/ml of NGF caused the prolongation of population doubling time of U-251MG/*trk* cells ($P < 0.005$). No such effect of NGF occurred with U-251MG or U-251MG/pRc cells. Subcutaneous tumor formation with U-251MG/*trk* cells was found in only two out of six SCID mice, although 100% transplantability was found with U-251MG or U-251MG/pRc cells. NGF-treated U-251MG/*trk* cells showed no transplantability in SCID mice.

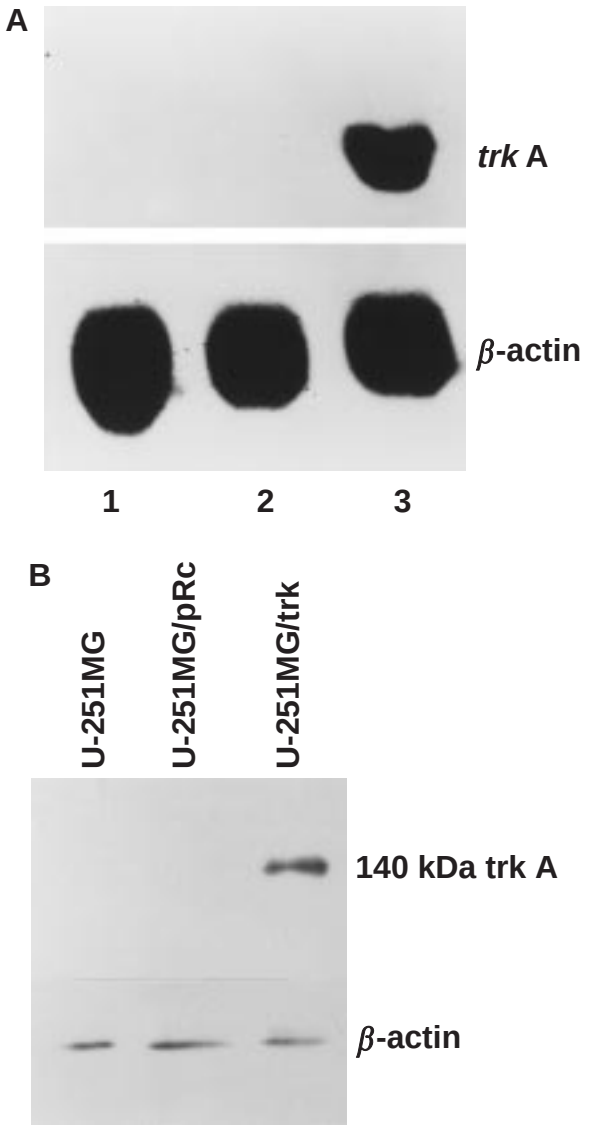

Figure 1. Northern blot of *trk* A mRNA (A) shows expression in U-251MG/*trk* (lane 3) but no expression in U-251MG (lane 1) or U-251MG/pRc (lane 2). Western blot of 140 kDa *trk* A proto-oncogene product (B) shows expression of U-251MG/*trk* The expression of β-actin was viewed for an internal protein and RNA controls.

Cell morphology

U-251MG/*trk* cells at the exponential growth phase (Figure 2E) showed the more elongated cellular processes than U-251MG (Figure 2A) and U-251MG/pRc (Figure 2C) cells. Furthermore, while U-251MG and U-251MG/pRc cells did not show any morphologic change with addition of 20 ng/ml NGF for 3 days (Figure 2B and D), NGF caused further elongation of cell processes in U-251MG/*trk* cells (Figure 2F) beyond that in U-251MG/*trk* cells without NGF treatment.

Table 1. Scatchard analysis for equilibrium binding of ^{125}I-NGF to cells

Cells	No. of binding sites/cell	Dissociation constant
U-251MG/*trk*	2.8×10^4	0.72×10^{-11} M
PC-12		
high-affinity	1.8×10^4	3.61×10^{-11} M
low-affinity	7.3×10^5	3.95×10^{-9} M
U-251MG	ND	ND
U-251MG/pRc	ND	ND

ND: no binding of NGF detected.

Expression of GFAP

Astrocytic differentiation of cells was analyzed by Western blot using anti-GFAP antibody (Figure 3). U-251MG and U-251MG/pRc cells showed the low level of expression of GFAP and no overexpression of GFAP was induced by treatment of these cells with 20 ng/ml NGF for three days. Expression of GFAP in U-251MG/*trk* cells was more abundant than that in U-251MG cells and also additionally overexpression of GFAP was induced by addition of NGF to U-251MG/*trk* cells. The level of β-actin was not different among all cell groups with or without NGF.

Cell-cycle analysis

Figure 4 and Table 3 show the cell-cycle pattern determined by a BrdU incorporation assay. The fractions of cells in G1 phase of the cycle in U-251MG and U-251MG/pRc cells were 34.62% and 29.21%, respectively and were not changed by treatment with NGF. 53.48% of U-251MG/*trk* cells were in the G1 fraction and the treatment with 20 ng/ml of NGF for 3 days further increase of the fraction of U-251MG/*trk* cells in the G1 phase (65.73%).

Tyrosine phosphorylation of trk *A and MAP kinases, and induction of* c-fos *mRNA by NGF in U-251MG/*trk

Tyrosine phosphorylation of *trk* A was observed in untreated U-251MG/*trk* cells, and increased

Table 2. Transplantabilities in SCID mice and population doubling times of U-251MG and its transfectants

Cells	Transplantability in population doubling	
	SCID mice	time (mean ± SD) (h)
U-251MG	6/6[#]	36.1 ± 3.68
U-251MG/pRc	6/6	37.9 ± 2.87
U-251MG/*trk*	2/6	52.1 ± 4.27[##]
U-251MG/*trk* treated with NGF	0/6	82.1 ± 4.21[###]

[#] No. of mice possibly transplanted/No. of total mice.
[##] Significantly different from U-251MG or U-251MG/pRc by Student's *t* test ($P < 0.05$).
[###] Significantly different from U-251MG/*trk* by Student's *t* test ($P < 0.005$).

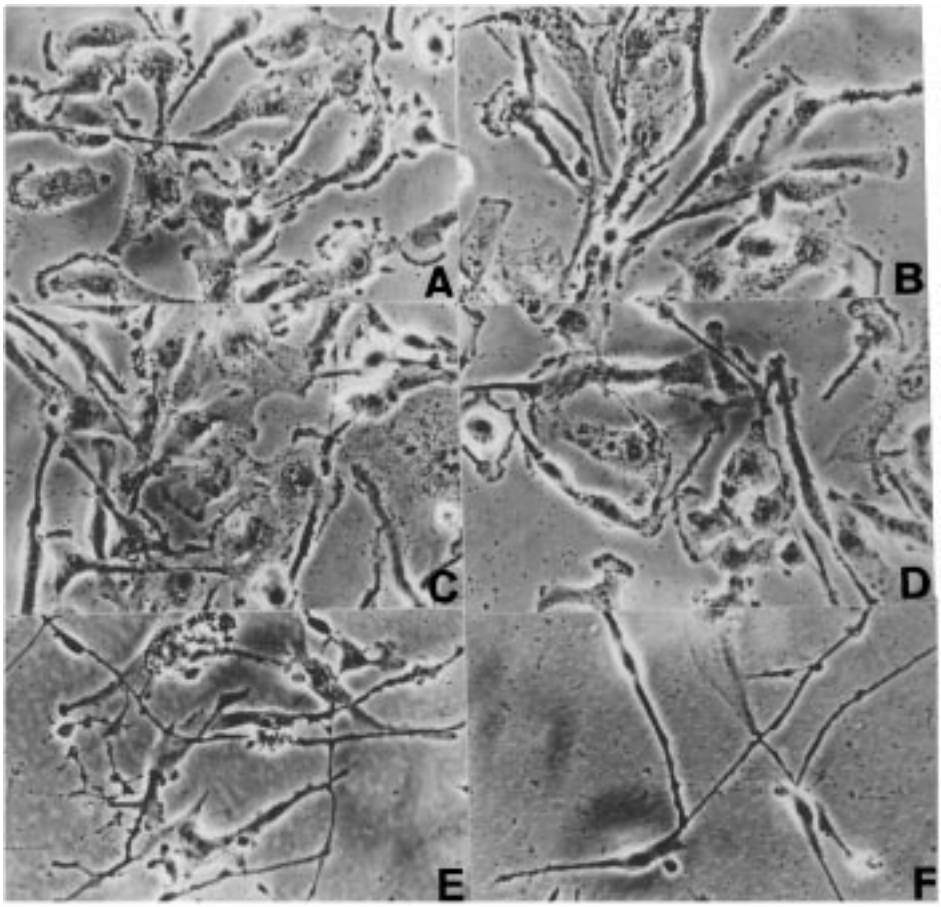

Figure 2. Morphology of U-251MG cells and its transfectants with or without 20 ng/ml NGF treatment for 3 days. Phase-contrast microscopy. (A) U-251MG without NGF treatment; (B) U-251MG with NGF treatment; (C) U-251MG/pRc without NGF treatment; (D) U-251MG/pRc with NGF treatment; (E) U-251MG/*trk* without NGF treatment: (F) U-251MG/*trk* with NGF treatment.

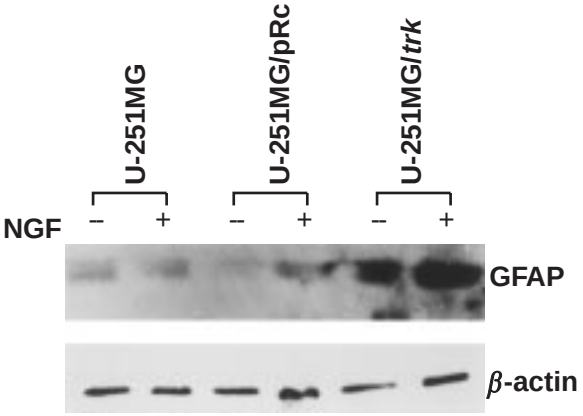

Figure 3. Changes of GFAP expression in U-251MG and its transfectants with or without 20 ng/ml NGF treatment for 3 days. The expression of β-actin was viewed for an internal protein controls.

phosphorylation was induced by 20 ng/ml NGF for 5 min (Figure 5A). The increased phosphorylation of *trk* A continued at 30 min after NGF treatment. Spontaneous phosphorylation of MAP kinases occurred in untreated U-251MG/*trk* cells, and 20 ng/ml of NGF induced increased phosphorylation of MAP kinases at 5 min after treatment. U-251MG cells with or without NGF treatment did not show MAP kinase phosphorylation (Figure 5B). The early response gene c-*fos* was upregulated transcriptionally in U-251MG/*trk* cells without NGF treatment and further upregulated at 5 min after 20 ng/ml of NGF (Figure 5C).

NGF production and NGF mRNA expression in human glioma cells

Five human glioma cell lines (U-251MG, U-251MG/*trk*, KNS-42, T-98G and A-172) were tested

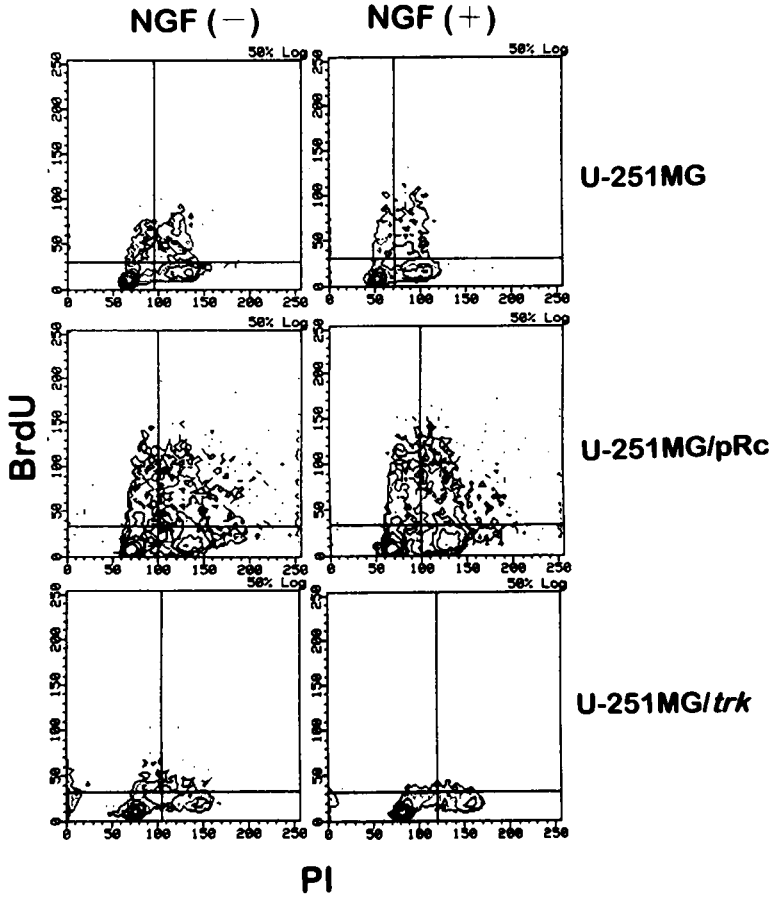

Figure 4. Cell-cycle pattern of U-251MG and its transfectants treatment with or without NGF according to a BrdU-incorporation assay. FITC fluorescence (BrdU) was displayed at the *y* axis versus propidium iodine (P1) fluorescence (DNA content) on the *x* axis.

Table 3. Cell-cycle analysis in U-251MG and its transfectants using BrdU incorporation assay

Cells	G1%	S%	G2M%
U-251MG			
NGF (−)	34.62	45.51	19.87
NGF (+)	33.65	43.02	21.65
U-251MG/pRc			
NGF (−)	29.21	45.18	25.61
NGF (+)	29.32	46.50	24.18
U-251MG/*trk*			
NGF (−)	53.48	32.30	14.22
NGF (+)	65.73	16.92	17.35

for the production and expression of NGF. NGF production in culture media was found in all glioma cell lines using sandwich ELISA (Figure 6A), especially in U-251MG, U-251MG/*trk* and T-98G cells, which produced high concentrations of NGF similar to the positive control, the C6 glioma cell line [25], which produced about 2.54 ng/ml/day of NGF. To examine if secreted NGF was biologically active, rat PC-12 pheochromocytoma cells were treated with concentrated culture media harvested from U-251MG cells. Within 12 h of NGF treatment, PC-12 cells extended neuritic projections (data not shown). Cellular NGF was expressed in U-251MG, U-251MG/*trk*, KNS-42 and T-98G cells by Western blot analysis (Figure 6B), and the NGF mRNA was expressed in U-251MG, U-251MG/*trk* and T-98G cells analyzed by RT-PCR (Figure 6C).

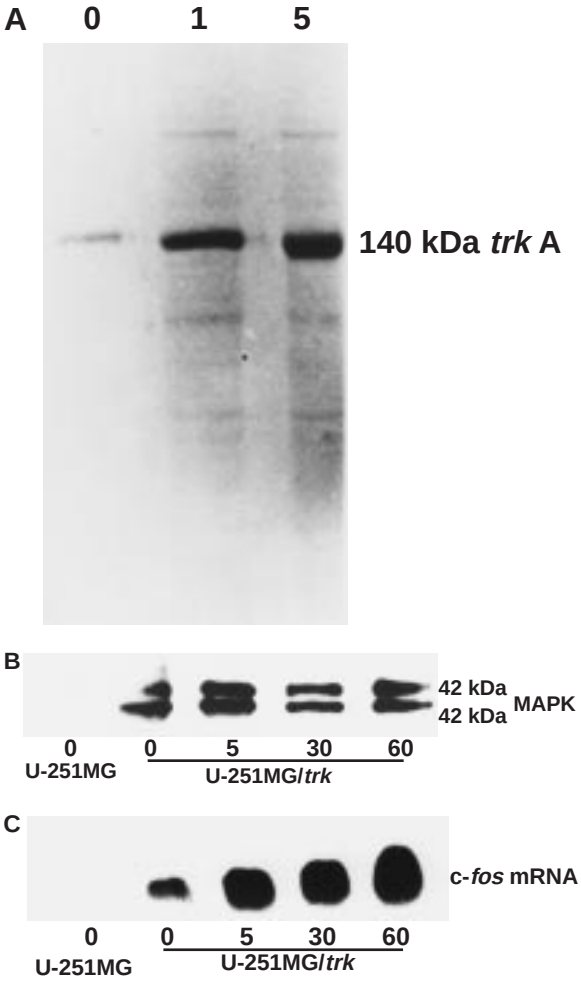

Figure 5. Tyrosine phosphorylation of *trk* A (A) and MAP kinase (B) were analyzed by Western blot using an anti-phosphotyrosine antibody in immunoprecipitation with anti-*trk* A antibody (A) and anti-MAP kinase antibody (B) with or without 20 ng/ml NGF treatment in U-251MG/*trk* cells. Changes of *c-fos* mRNA expression (C) under the treatment with NGF were analyzed using Northern blot. U-251MG cells without NGF treatment were used as a control. The numbers 0, 1 and 5 in Figure 5A, and the numbers 0, 5, 30 and 60 in Figure 5B and C refer to the minutes after NGF treatment.

Discussion

Our study has important implications regarding the role in cell growth and differentiation of *trk* A proto-oncogene transfected into human glioma cells. When the human *trk* A cDNA was transfected into a human glioma cell line (U-251MG) which lacks expression of both endogenous *trk* A and low-affinity NGF receptor, the transfectant expressed the exogenous *trk* A mRNA and functional high-affinity NGF receptor. Transfection of *trk* A cDNA caused the partial induction of cell differentiation, G1 arrest, growth inhibition, tyrosine phosphorylation of *trk* A proto-oncogene product and activation of MAP kinase, and the exogenous NGF caused further induction of terminal differentiation and growth inhibition. Endogenous NGF secreted from glioma cells therefore is considered important in the induction of differentiation by transfer of exogenous *trk* A cDNA.

Growth inhibition, cell-cycle inhibition, decrease in tumorigenicity, and induction of differentiation

High-affinity NGF binding sites were reconstituted by transfection of human *trk* A cDNA into human

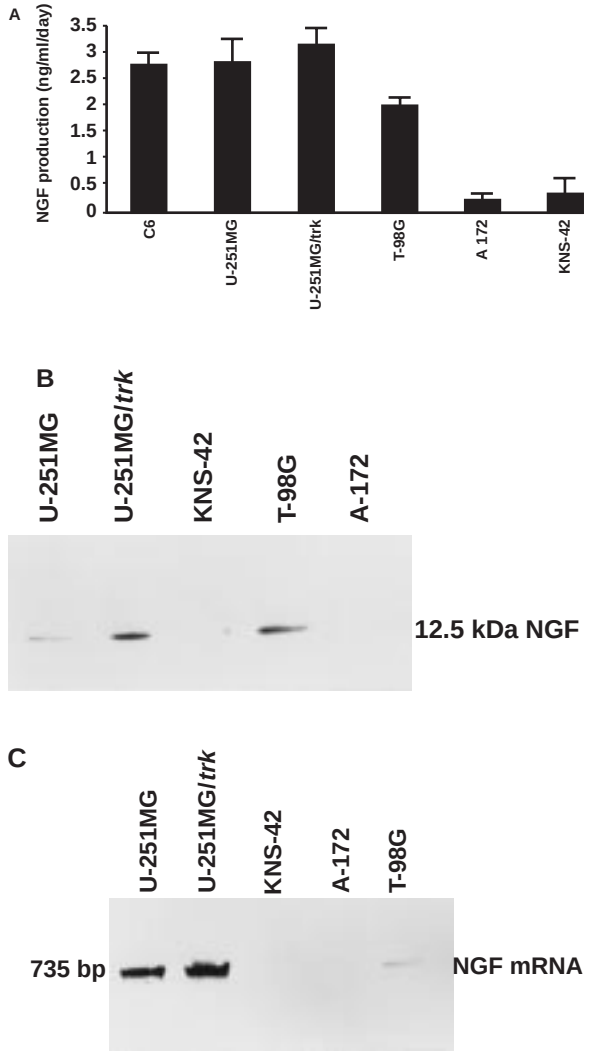

Figure 6. Expression of NGF in five human glioma cell lines as shown by Western blot (B) and RT-PCR (C). Production of NGF by human glioma cell lines (A) was analyzed by sandwich ELISA. A rat glioma cell line, C6, was used as a positive control for NGF production.

glioma cells not expressing endogenous *trk* A proto-oncogene or low-affinity NGF receptor. Furthermore, transfection of *trk* A proto-oncogene into glioma cells caused growth inhibition, decreased tumorigenicity in SCID mice, and cell accumulation in the G1 state. These growth inhibitory effects from transfection of *trk* A cDNA were correlated with induction of differentiation.

The expression of GFAP is a defining characteristic of astrocytes [26], and GFAP content and the elongation of cellular processes may indicate magnitude of astrocytic differentiation in glioma cells [27]. In this study, astrocytic differentiation of glioma cells was analyzed by morphologic changes and expression of GFAP. The transfectant with exogenous high-affinity NGF receptor showed the differentiated phenotype in contrast to the parent cells. Differentiating cells manifest a prolonged G1 phase and inhibition of G1/S transition [28,29]. Reportedly, addition of NGF to PC-12 cells at the exponentially growing phase results in cell accumulation in the G1 state. Rudkin et al. [30] found that in an exponentially growing population, 50% of PC-12 cells were in G1, while exposure to 50 ng/ml of NGF resulted in the accumulation of 70% of cells in G1. The present report showed a G1-phase arrest of exponentially growing U-251MG cells with transfection of *trk* A cDNA, increased further by treatment with NGF.

Tyrosine phosphorylation of trk *A and MAP kinases, and induction of c-*fos *mRNA by NGF in U-251MG/*trk

Binding of NGF to *trk* A produces biologic responses through activation of the tyrosine-kinase domain, resulting in rapidly increased phosphorylation of certain cellular substrates such as MAP kinases [31] and in stimulation of multiple second messenger pathways [32]. Stimulation of these pathways causes a series of post-translational modifications of specific proteins, which lead to immediate changes in the cellular phenotype [32]. Tyrosine phosphorylation of *trk* A by NGF is rapid and specific, and occurs with only picomolar quantities of NGF [33]. Kaplan et al. [33] showed that tyrosine phosphorylation was half-maximal at 0.1 ng/ml NGF, indicating that *trk* phosphorylation occurs at physiologically relevant NGF concentrations.

NGF treatment of PC-12 cells leads to activation of numerous genes that have been classified into two major sets based on the kinetics of their response: immediate-early genes and late response genes [32]. The immediate-early genes are believed to be critical to NGF action, as many of them, including c-*fos*, encode transcription factors that may regulate of transcription of late response genes [34]. The kinetics of c-*fos* induction are rapid and transient, with gene transcription beginning within 5 min of the stimulus, peaking at 15–30 min, and returning to basal levels within 1 h [34]. Expression of c-*fos* has been induced with as little as 1 ng/ml NGF, with the level of induction increasing

steadily with increasing amounts of NGF up to 10–100 ng/ml, where transcription reaches a plateau [35].

Miyasaka et al. [36] reported that phosphorylation of MAP kinase depended on the concentration of NGF, with the NGF concentration for half-maximal stimulation of MAP kinase in PC-12 cells being 0.3 nM (3.75 ng/ml), and the maximally effective concentration being 1 nM (12.5 ng/ml).

These results indicated that stimulation of signal transduction through *trk* A was induced by picomolar quantities of NGF [33]. In the present study, transfectant with *trk* A cDNA without exogenous NGF showed autophosphorylation of the tyrosine residue in the *trk* A proto-oncogene product, activation of MAP kinase and upregulation of c-*fos* mRNA. This phenomenon was mediated by exogenous high-affinity NGF receptor, because the parent U-251 MG cells did not respond to NGF. We believe that NGF produced by glioma cells might be the ligand for the exogenous high-affinity NGF receptor which stimulated the signal transduction pathway.

NGF production and NGF mRNA expression in human glioma cells

Induction of growth inhibition and differentiation of glioma cells by transfection of *trk* A cDNA in the present study suggests endogenous NGF production by the glioma cells. Although the main sites of NGF expression in the central nervous system are specific populations of neurons [37], cultured rat glial cells [38,39], human glial cells [40], and also glioma cells [25] synthesize NGF protein and mRNA. It has been reported that the rate of NGF secretion by mouse astroglial cells is about 5 pg/h/10^5 cells [41]. Our study demonstrated that all human glioma cell lines expressed the 12.5 kDa mature NGF [24,42] and secreted active NGF into culture media, at a sufficient rate in U-251MG and U-251MG/*trk* (3 ng/ml/24 h) to induce differentiation and growth inhibition of U-251MG/*trk*. Additionally, endogenous NGF secreted from U-251MG/*trk* cells bound to functional high-affinity NGF receptor, which activated the *trk* A tyrosine kinase and MAP kinases. This induced c-*fos* mRNA overexpression resulting in cell differentiation and growth arrest. Further, exogenous NGF brought about terminal differentiation of glioma cells transfected with *trk* A cDNA.

Conversion of malignant cells into terminally differentiated postmitotic cells is a rare event. Introduced into neuroblastoma cells and neurogenic cells *trk* A cDNA has suppressed the tumorigenic phenotype [13,15,43,44]. Poluha et al. [15] have reported that *trk* A-transfected SH-SY5Y cells ceased proliferation and irreversibly differentiated under the treatment with NGF. Matsushima and Bogenmann [13] have demonstrated that transfection of human *trk* A cDNA into a neuroblastoma cell line (HLTA230) led to growth arrest and cell differentiation with NGF stimulation *in vivo* and *in vitro*.

The transfection of *trk* A into an immortalized rat hypothalamic cell line, GT-101, induced neuronal differentiation and a rapid transient expression of c-*fos* mRNA with NGF stimulation [44]. These results suggest that *trk* A transfection of neuronal and neuroblastoma cells can be used to further elucidate NGF signaling.

In conclusion, the present results demonstrated that constitutive expression of human *trk* A cDNA in malignant glioma cells could reconstitute a functional NGF–NGF receptor signal cascade and an extracellular environment conducive to terminal differentiation of glioma cells secreting NGF.

References

1. Schoenberg BS: The epidemiology of central nervous system tumors. In: Walker MD (ed). Oncology of the Nervous System, Boston, Martinus Nijhoff, 1983 pp 1–30
2. Mahaley MS, Mettlin C, Natarajan N, Laws ER, Peace BB: National survey of patterns of care for brain-tumor patients. J Neurosurg 71: 826–836, 1989
3. Huber BE: Gene therapy strategies for treating neoplastic disease. Ann NY Acad Sci 716: 6–11, 1992
4. Levi-Montalcini R: The nerve growth factor 35 years later. Science 237: 1154–1162, 1987
5. Yankner BA, Shooter EM: The biology and mechanism of action of nerve growth factor. Annu Rev Biochem 51: 845–868, 1982
6. Johnson D, Lanahan A, Buck CR, Sehgel A, Morgan C, Mercer E, Bothwell NM, Chao M: Expression and structure of the human NGF receptor. Cell 47: 545–554, 1986
7. Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM: Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325: 593–597, 1987
8. Kaplan D, Hempsteadt B, Martin-Zanca D, Chao M, Parada L: The *trk* proto-oncogene product: A signal transducing receptor for nerve growth factor. Science 252: 554–558, 1991
9. Klein R, Jing S, Nanduri V, O'Rouke E, Barbacid M: The *trk* proto-oncogene encodes a receptor for nerve growth factor. Cell 65: 189–197, 1991
10. Barbacid M: Nerve growth factor: A tale of two receptors. Oncogene 8: 2033–2042, 1993

11. Hempstead B, Schleifer LS, Chao M: Expression of functional nerve growth factor receptors after gene transfer. Science 243: 373–375, 1989
12. Suzuki T, Bogenmann E, Shimada H, Stram D, Seeger RC: Lack of high-affinity nerve growth factor receptors in aggressive neuroblastomas. J Natl Cancer Ins 85: 377–385, 1993
13. Matsushima H, Bogenmann E: Expression of *trk* A cDNA in neuroblastomas mediates differentiation *in vitro* and *in vivo*. Mol Cell Biol 13: 7447–7456, 1993
14. Nakagawara A, Arima M, Azar CG, Scavarda NJ, Brodeur GM: Inverse relationship between *trk* expression and *N-myc* amplification in human neuroblastomas. Cancer Res 52: 1364–1368, 1992
15. Poluha W, Poluha DK, Ross AH: *Trk* A neurogenic receptor regulates differentiation of neuroblastoma cells. Oncogene 10: 185–189, 1995
16. Kokunai T, Kawamura A, Tamaki N: Functional analysis of *trk* proto-oncogene product in medulloblastoma cells. Neurol Med Chir 36: 796–804, 1996
17. Martin-Zanca D, Hughes SH, Barbacid MA: Human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences. Nature 319: 743–748, 1986
18. Maniatis T, Fritsch EF, Sambrook J: Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, New York, 1982
19. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685, 1970
20. Sutter A, Riopelle RJ, Hanis-Warrick RM, Shooter EM: Nerve growth factor receptors. J Biol Chem 254: 5972–5982, 1979
21. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76: 4350–4354, 1979
22. Vale RD, Shooter EM: Assaying binding of nerve growth factor to cell surface receptors. Methods Enzymol 109: 21–39, 1985
23. Stewart N, Hicks GC, Paraskevas F, Mowat M: Evidence for a second cell cycle block at G2/M by p53. Oncogene 10: 109–115, 1995
24. Barnett J, Baecker P, Routledge-Ward C, Bursztyn-Pettegrew H, Chow J, Nguyen B, Bach C, Chan H, Tuszynski MH, Yoshida K, Rubalcava R, Gage FH: Human β nerve growth factor obtained from a baculovirus expression system has potent *in vitro* and *in vivo* neurotrophic activity. Exp Neurol 110: 11–24, 1990
25. Mocchetti I, De Bernardi MA, Szekely AM, Alho H, Brooker G, Costa E: Regulation of nerve growth factor biosynthesis by β-adrenergic receptor activation in astrocytoma cells: A potential role of c-*Fos* protein. Proc Natl Acad Sci USA 86: 3891–3895, 1989
26. Bongeam-Rudloff E, NistÈr M, Betsholtz C, Wang J-L, Stenman G, Huebner K, Croce CM, Westermark B: Human glial fibrillary acidic protein: Complementary DNA cloning, chromosome localization, and messenger RNA expression in human glioma cell lines of various phenotypes. Cancer Res 51: 1553–1560, 1991
27. Weinstein DE, Shelanski ML, Liem RHH: Suppression by antisense mRNA demonstrates a requirement for the glial fibrillary acidic protein in the formation of stable astrocytic processes in response to neurons. J Cell Biol 112: 1205–1213, 1991
28. Chen P-L, Scully P, Shew J-Y, Wang JYL, Lee W-H: Phosphorylation of the retinoblastoma gene product is modulating during the cell cycle and cellular differentiation. Cell 58: 1193–1198, 1989
29. Kokunai T, Kawamura A, Tamaki N: Induction of differentiation by wild-type p53 gene in a human glioma cell line. J Neuro-Oncol 32: 125–133, 1996
30. Rudkin BB, Lazarovici P, Levi B-Z, Abe Y, Fujita K, Guroff G: Cell cycle-specific action of nerve growth factor in PC12 cells: Differentiation without proliferation. EMBO J 8: 3319–3325, 1989
31. Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH, Yancopoulos GD: ERKs: A family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 65: 663–675, 1991
32. Halegoua S, Armstrong RC, Kremer NE: Dissecting the mode of action of a neuronal growth factor. Curr Top Microbiol Immunol 165: 119–170, 1991
33. Kaplan DR, Martin-Zanca D, Parada LF: Tyrosine phosphorylation and tyrosine kinase activity of the *trk* proto-oncogene product induced by NGF. Nature 350: 158–160, 1991
34. Morgan JI, Curran T: Stimulus-transcription coupling in the nervous system: Involvement of the inducible proto-oncogenes *fos* and *jun*. Annu Rev Neurosci 14: 421–451, 1991
35. Greenberg ME, Greene LA, Ziff EB: Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC 12 cells. J Biol Chem 260: 14101–14110, 1985
36. Miyasaka T, Sternberg DW, Miyasaka J, Sherline P: Nerve growth factor stimulates protein tyrosine phosphorylation in PC-12 pheochromocytoma cells. Proc Natl Acad Sci USA 88: 2653–2657, 1991
37. Zafra F, Lindholm D, CastrÈn E, Hartikka J, Thonen H: Regulation of brain-derived neurotrophic factor and nerve growth factor mRNA in primary cultures of hippocampal neurons and astrocytes. J Neurosci 12: 4793–4799, 1992
38. Spranger M, Lindholm D, Bandtlow C, Heumann R, Gnahn H, Näher-NoÈ M, Thoenen H: Regulation of nerve growth factor (NGF) synthesis in the rat central nervous system: Comparison between the effects of interleukin-1 and various growth factors in astrocyte cultures and *in vivo*. Eur J Neurosci 2: 69–76, 1990
39. Yamakuni T, Ozawa F, Hishinuma F, Kuwano R, Takahashi Y, Amano Y: Expression of β-nerve growth factor mRNA in rat glioma cells and astrocytes from rat brain. FEBS Lett 223: 117–121, 1987
40. Norrgren G, Ebendal M, Belew M, Jacobson C-O, Porath J: Release of nerve growth factor by human glial cells in culture. Exp Cell Res 130: 31–39, 1980
41. Furukawa S, Furukawa Y, Satoyoshi E, Hayashi K: Synthesis and secretion of nerve growth factor by mouse astroglial cells in culture. Biochem Biophys Res Comm 136: 57–63, 1986

42. Edwards RH, Selby MJ, Mobley WC, Weinrich SL, Hruby DE, Rutter WJ: Processing and secretion of nerve growth factor: Expression in mammalian cells with a vaccinia virus vector. Mol Cell Biol 8: 2456–2464, 1988
43. Bogenmann E, Torres M, Matsushima H: Constitutive N-*myc* gene expression inhibits *trk* A mediated neuronal differentiation. Oncogene 10: 1915–1925, 1995
44. Zhou J, Holtzman DM, Weiner RI, Mobley WC: Expression of *Trk* A confers neuron like responsiveness to nerve growth factor on an immortalized hypothalamic cell line. Proc Natl Acad Sci USA 91: 3824–3828, 1994

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