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## Original Paper

# Activation of trk-A but not trk-B Signal Transduction Pathway Inhibits Growth of Neuroblastoma Cells

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**In neuroblastoma tumours, the expression of high levels of trk-A mRNA, which encodes the high-affinity nerve growth factor (NGF) receptor, is associated with good prognosis. Constitutive expression of brain-derived neurotrophic factor (BDNF) and variable expression of its receptor trk-B are frequently detected in tumours from patients with a poor prognosis. To evaluate the biological consequences of activation of the trk-A or trk-B signal transduction pathways in neuroblastoma cells, the *trk-A* or *trk-B* gene was transfected into the trk negative 15N neuroblastoma cell line. Clones expressing *trk-A* or *trk-B* were treated with specific ligands and evaluated for growth and differentiation. Both ligands induced neurite extension. Treatment of the 15N-*trk-A* clones with NGF inhibited proliferation (80–90% decrease), while treatment of the 15N-*trk-B* clone with BDNF had no effect (< 10% decrease). NGF-induced growth inhibition was concentration dependent. Such studies indicate that differential trk expression may affect the biology of neuroblastoma tumours and contribute to differences in the clinical course of patients.** © **1997 Elsevier Science Ltd.**

**Key words: neuroblastoma, trk-A, trk-B, NGF, BDNF**
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## INTRODUCTION

NEUROTROPHINS (Nts) are a family of neurotrophic factors that play important roles in the development of both the peripheral and central nervous systems. These agents have several functions: they are survival factors; promote differentiation of the different classes of neurons; and play a role in the guidance of neuronal process outgrowth. Nts' actions are mediated by the interaction of two types of receptors. Nts bind to a family of tyrosine kinase receptors named trks and to the transmembrane glycoprotein gp75. Among the best characterised Nts are NGF and BDNF, which bind with high affinity to trk-A and trk-B. There are two major forms of trk-B: gp145trk-B, which encodes for the trk-B full-length tyrosine kinase receptor, and gp95trk-B, which lacks the tyrosine kinase domain. Two forms of full-length trk-A, gp140trk-A are known [1].

Neuroblastoma is a neural crest derived paediatric tumour arising from neural crest precursors. The remarkable clinical and biological heterogeneity shown by neuroblastoma suggests that its biology is closely related with the developmental stage of the neurons from which the tumour originates. *In situ* hybridisation studies have shown differential trk mRNA expression in neurons during development. Several studies showing a relationship between trk mRNA expression in neuroblastoma tumours and patient survival have urged the need for a better understanding of the regulation and function of Nts and trk genes.

Several groups have found the expression of high levels of trk-A to be correlated with good prognosis [2–7], while constitutive expression of BDNF and variable expression of trk-B are associated with poor prognosis [8, 9]. Many human neuroblastoma cell lines constitutively express trk-A, but are not responsive to NGF stimulation [10] and few cell lines express trk-B. In order to gain further information about the action of Nts on neuroblastoma cells, trk receptors were introduced into neuroblastoma by transfection, or drugs were used to regulate selectively trk receptors expression. It has been shown that trk-A has a function in growth arrest and differentiation

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of neuroblastoma cells [9, 11, 12]. Activation of the BDNF-trk-B signal transduction promoted cell survival and neurite outgrowth [8, 13–15]. In addition, activation of BDNF-trk-B signal transduction in SY5Y cells, induced to express trk-B by retinoic acid, revealed that BDNF stimulated neuroblastoma cell survival, disaggregation and invasion [15]. Interestingly, these are all characteristics of metastatic cells.

*In situ* hybridisation studies have shown that trk mRNA increases during embryonal life and decreases in most tissues after birth. It is possible that trk expression and the biology of the neuroblastoma cell line is correlated with the developmental stages of the neurons from which the tumours originated. Therefore, to understand fully Nts-trk signal transduction, it is important to study their interaction in a single cell line. In this study, the effects of the activation of trk-A and trk-B signal transduction were compared by transfecting the receptors into the LA-1-15N cell line. This cell line is a good experimental model because it lacks both gp140trk-A and gp145trk-B expression [13].

## MATERIALS AND METHODS

*Cell culture*

The neuronal subclone of the human neuroblastoma cell line LA-1-15N (15N) was cultured in RPMI-1640 containing 10% fetal calf serum (Biofluid, Rockville, Maryland, U.S.A.). Cells were treated with indicated concentrations of NGF (UBI, Lake Placid, New York, U.S.A.), BDNF (Promega, Madison, Wisconsin) or control solvent for the indicated times.

*Transfection*

15N cells were electroporated with 10 mg of vector pIRV Neo SV containing human *trk-A*, rat *trk-B* cDNA or the vector alone *(WT)* at 250 V, 960 mF capacitance. Cells were selected with 300 mg/ml geneticin (Gibco BRL, Grand Island, New York, U.S.A.).

*Cell growth assay*

15N cells were plated at a density of $2.5 \times 10^3$ cells per well in 96-well flat-bottom plates. At the indicated time, cells were labelled with 1 mCi of [$^3$H]thymidine per well for 18 h and harvested with PHD cell harvester (Cambridge Tech., Inc., Watertown, Massachusetts, U.S.A.). Radioactivity was measured in an LS 1801 liquid scintillation counter (Beckman Instruments Inc., Fullerton, California, U.S.A.).

## RESULTS

*Trk-A and trk-B transection of* 15*N cells*

In order to test the biological function of trk-A and trk-B in neuroblastoma these two receptors were transfected in LAN-1-15N (15N), a cell line that has been previously described by Kaplan [13] to lack expression of both receptors. 15N cells were transfected with an expression vector containing the human *trk-A* gene, the rat *trk-B* gene or the vector alone, using electroporation as previously described [13]. Expression of gp140trk-A and gp145trk-B proteins were confirmed in Western blot experiments, using antibodies that specifically recognise these proteins. Several clones expressed gp140trk-A and one (15N-trk-B) expressed gp145trk-B. All the stable *trk* transfected clones exhibited receptor autophosphorylation, induction of early response genes and neurite outgrowth after neurotrophin stimulation. Cells transfected with the control vector did not exhibit any of the beforementioned phenomena upon Nts stimulation.

*Table* 1. *Effect of neurotrophin treatment on the growth of* trk-A *and* trk-B *transfected neuroblastoma cells*

| | NGF | BDNF |
|---|---|---|
| 15N-*WT* | 100% | 96% |
| 15N-*trk-A* | 7% | — |
| 15N-*trk-B* | — | 95% |

DNA replicative activity is tested by [$^3$H]thymidine uptake and results are expressed as percentage of control solvent treated cells.

*NGF treatment inhibits* 15*N-trk-A cell growth*

To test whether Nts stimulation had an effect on the growth of *trk* transfected cells, 15N-*trk-A* clones were treated with 100 ng/ml NGF; 15N-*trk-B* clones with 100 ng/ml of BDNF; and the 15N-*WT* clone was treated with both NGF and BDNF. [$^3$H]dThymidine incorporation was tested after 7 days. Since NGF has a similar effect on growth in all five 15N-*trk-A* cell lines tested, data on one representative cell line are shown. In the 15N-*trk-A* clone, NGF caused a 93% decrease in growth (Table 1). BDNF treatment of the 15N-*trk-B* clone had no effect on growth. Although trk-A and trk-B induce neurite outgrowth, these results indicate that upon the activation of these receptors there is a differential effect on cell growth.

*Effect of different NGF concentration on* 15*N*-trk-A *transfected cells*

The effect of different concentrations of NGF was tested on the 15N-*trk-A* clonal cell line. 15N-*trk-A* cells were treated either with 0.1, 1, 5, 10, 100 ng/ml of NGF or control solvent. [$^3$H]dThymidine incorporation was tested after 7 days. NGF-stimulated growth inhibition was concentration dependent. Concentrations as low as 0.1 ng/ml were sufficient to obtain a 17% decrease in cell growth. NGF concentrations between 1 and 5 ng/ml inhibited growth, respectively, by 41% and 75% (Table 2). Thus, NGF inhibited growth of 15N-trk-A transfected cells at concentrations that are physiologically significant.

## DISCUSSION

Our study provides further evidence of the function of trk receptors in the biology of neuroblastoma tumours. The transfection of these receptors in the same cell line provides us with a model to compare the effects of the activation of trk-A and trk-B. We showed that activation of NGF/trk-A signal transduction leads to growth inhibition (data not shown). In contrast to the NGF/trk-A pathway, activation of the BDNF/trk-B pathway leads to differentiation but not

*Table* 2. *Effect of different NGF concentrations on the growth of* trk-A *transfected cells*

| NGF (ng/ml) | cpm | (% decrease) |
|---|---|---|
| 0 | 60 129 ± 8367 | (—) |
| 0.1 | 50 079 ± 13 006 | (17) |
| 1 | 35 233 ± 7038 | (41) |
| 5 | 15 221 ± 3327 | (75) |
| 10 | 8536 ± 3899 | (86) |
| 100 | 802 ± 583 | (99) |

DNA replicative activity was evaluated by [$^3$H]thymidine uptake and results are expressed in cpm.

growth arrest. Our results imply that even though these two receptors can equally induce neuritogenesis, they differ in their capacity to inhibit growth.

Results obtained in 15N cells are consistent with what was previously found in the literature. *trk-A* transfection blocks growth and induces neuritogenesis [9, 11, 12]. The lack of trk-B involvement in growth inhibition is consistent with data which found that many aggressive neuroblastomas, especially those with *MYCN* amplification, express both full-length trk-B and BDNF [8, 12]. The trk-B results are also consistent with what was previously studied in SY5Y cells, in which activation of the trk-B signal transduction stimulated characteristics of metastatic cells [15]. Furthermore, trk-A-mediated growth arrest is induced by nanomolar concentrations that are in the physiological range. We have also previously shown that these concentrations successfully trigger trk receptor autophosphorylation [15].

Our results also show that upon transfection of *trk-A* or *trk-B*, 15N cells become responsive to NGF or BDNF. This result is consistent with this cell line having a functional signal transduction mechanism. A functional signal transduction of a typical tyrosine kinase receptor consists of the capacity of the receptor to communicate that it has been bound by the ligand. This signal transduction is mediated through a sequence of phosphorylation of proteins. It starts with autophosphorylation of the receptor and terminates with the execution of a complex programme of transcriptional events. Recent literature has partially clarified how the pheochromocytoma cells PC-12 discriminate between a mitotic and differentiative signal, showing a different pattern and different kinetics of protein phosphorylation [16]. It is also likely that the difference in trk-A and trk-B function is mediated through differences in signal transduction.

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