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Expression of low-affinity NGF receptor and trkB mRNA in human SH-SY5Y neuroblastoma cells

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We have used the human neuroblastoma cell line SH-SY5Y as a model system to investigate the expression and regulation of the receptors for brain-derived neurotrophic factor (BDNF), a member of the nerve growth factor (NGF) family of neurotrophins. We demonstrate that SH-SY5Y cells express transcripts encoding the low-affinity NGF receptor (LNGFR) and trkB, the signal transducing receptor unit for BDNF. Interaction of BDNF with SH-SY5Y cells increased the transcription of the c-fos gene, showing that these molecules encode functional BDNF receptors. Our findings that differentiating agents such as retinoids and cAMP analogs increased the expression of LNGFR, but decreased trkB mRNA levels, suggest that LNGFR and trkB have different roles during neuronal differentiation.

Brain-derived neurotrophic factor; Polymerase chain reaction; Gene expression; Differentiation

1. INTRODUCTION

Neurotrophins play an essential role in the growth, survival and differentiation of neurons in both the peripheral and central nervous system. Brain-derived neurotrophic factor (BDNF) [1] is a member of the nerve growth factor (NGF) [2] family of structurally related neurotrophins, which also includes neurotrophin-3 [3] and neurotrophin-4/5 [4,5]. BDNF has been shown to promote the survival of embryonic neurons in the nodose ganglia and dorsal root ganglia in vivo [6,7] and of retinal ganglion cells [8], basal forebrain cholinergic neurons [9,10] and ventral mesencephalic dopaminergic neurons [10,11] in vitro. Recent studies have demonstrated that BDNF is also a trophic factor for motor neurons [12,13].

The effects of neurotrophins are mediated by their interaction with specific receptors on target cells. All neurotrophins bind to a low-affinity receptor (designated here as LNGFR for low-affinity NGF receptor) with similar affinities in the nanomolar range. The protein tyrosine kinases trk, trkB and trkC have been shown to be components of the high-affinity neurotrophin receptors and to mediate functional responses to neurotrophins [14]. Recent studies have established that trkB is an essential component of the high-affinity receptor for BDNF and is phosphorylated on tyrosine after exposure to BDNF [15–18]. Activation of trkB by BDNF leads to differentiation and proliferation of mouse 3T3 fibroblasts [15,16] and rat pheochromocytoma PC12 cells [18] into which the trkB gene has been transfected. As demonstrated by in situ hybridization, trkB is expressed at multiple sites in the peripheral and central nervous systems [19,20].

BDNF protects cultured dopaminergic neurons and the human dopaminergic neuroblastoma cell line SH-SY5Y against the neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine, a chemical known to cause Parkinson's disease in humans [11,21]. This suggests that dopaminergic neurons and SH-SY5Y cells contain the essential components for binding and responding to BDNF.

In the present study, we have used cultured SH-SY5Y neuroblastoma cells as a model to investigate the expression and regulation of BDNF receptors. We analyzed whether treatment with differentiation agents modulates the expression of the BDNF receptors. Using reverse transcription combined with the polymerase chain reaction (RT-PCR), we found that SH-SY5Y cells express LNGFR and trkB transcripts and functional BDNF recepetors. Furthermore, we demonstrate that long-term exposure to differentiating agents, such as retinoids and cAMP analogs, regulates LNGFR and trkB mRNA expression in opposite ways.

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Abbreviations. BDNF, brain-derived neurotrophic factor; dbcAMP, dibutyryl cyclic AMP, FCS, fetal calf serum; LNGFR, low-affinity NGF receptor, NGF, nerve growth factor; RT-PCR, reverse transcription-polymerase chain reaction.

2. MATERIALS AND METHODS

2.1. *Materials*

Vitamin A (retinol) and dibutyryl cyclic AMP (dbcAMP) were purchased from Sigma Restriction endonucleases were obtained from

Boehringer Mannheim. Tretinoin (All-*trans*-retinoic acid), isotretinoin (13-*cis*-retinoic acid; Ro 4-3780) and acitretin (All- *trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8- nonatetraenoic acid; Ro 10-1670) were kindly provided by Dr. W. Bollag (F. Hoffmann-La Roche, Basel, Switzerland). Human recombinant BDNF was a gift from Dr. L. Aloe (Institute of Neurobiology, CNR, Rome, Italy) The human neuroblastoma cell line SH-SY5Y was obtained from Dr. J. Biedler (Sloan Kettering Cancer Research Institute, New York, NY, USA).

2 2. *Cell culture*

SH-SY5Y cells were maintained in supplemented RPMI 1640 (Seromed) containing 10% fetal calf serum (FCS, Gibco) at 37°C in humidified 5% CO_2/95% air as described earlier [22]. SH-SY5Y cells were stimulated as indicated in the respective experiments. During stimulation, cells were kept in RPMI 1640 with 1% FCS Stock solutions of retinoids were prepared in 0 1% dimethyl sulfoxide and stored in the cold protected from light. Control cultures were treated with an equivalent volume of solvent.

2.3. *RNA preparation*

Total cellular RNA was isolated by acid guanidinium thiocyanate/phenol/chloroform extraction [23] and quantified spectrophotometrically by absorbance at 260 nm. The integrity of RNA was checked by formaldehyde/agarose gel electrophoresis.

2.4. *Reverse transcription-polymerase chain reaction (RT-PCR)*

Total RNA of each sample was first reverse-transcibed into cDNAs, which in turn were subjected to PCR amplification using primers specific for LNGFR [24], trkB [19] and c-fos [25] (see below) essentially as described previously [26] PCR amplification of a constitutively expressed control mRNA encoding the ribosomal protein S12 [27] was used as a measure of the amount of input RNA. Controls using RNA samples without RT or controls without RNA were used to demonstrate absence of contaminating DNA. In brief, cDNA was synthesized in a final volume of 20 μl with the following components: 1 μg total RNA, 50 mM Tris-HCl pH 8.3, 3 mM $MgCl_2$, 75 mM KCl, 10 mM dithiothreitol, 1 mM dNTPs, 100 pmol of random hexamer oligonucleotides (Pharmacia), 20 units of RNase inhibitor (Boehringer Mannheim) and 200 units of Moloney Murine Leukemia Virus reverse transcriptase (Bethesda Research Laboratories). The mixture was incubated at 37°C for 60 min, followed by 5 min at 95°C and flash cooling to 4°C.

PCR was performed in a total volume of 15μl containing cDNA made from 15 ng of total RNA, 18 mM Tris-HCl pH 8.3, 55 mM KCl, 0.08 mg/ml gelatine, $MgCl_2$ either 1.8 mM for LNGFR and trkB or 1.2 mM for S12, 0.2 mM dNTPs, 0.4 μM each 5'- and 3'-primers and 0.4 unit Taq DNA polymerase (Boehringer Mannheim) The PCR mixture was overlaid with mineral oil (Sigma) and incubated in an Ericomp thermal cycler. The amplification steps involved denaturation at 94°C for 1 min, annealing for 2 min at 55°C with LNGFR primers or 50°C with trkB and S12 primers, and extension at 72°C for 3 min. Coamplification of c-fos and S12 was performed as above with 1 mM $MgCl_2$ at an annealing temperature of 55°C. Samples (4μl) of the PCR mixtures were analyzed by electrophoresis in 1.3% agarose gels in the presence of ethidium bromide (0.5 μg/ml), followed by alkaline blotting of the fragments onto nylon membranes and subsequent hybridization with specific digoxigenin-labeled DNA probes [28]. Detection was with AMPPD as chemiluminescent substrate for alkaline phosphatase conjugated to anti-digoxigenin antibodies (Boehringer Mannheim), as indicated by the manufacturer. Appropriate exposures of Kodak X-Omat films were quantified using a video densitometer (Model 620, Bio-Rad). The results were expressed as the ratio of the signal of amplified LNGFR, trkB or c-fos to the signal of amplified ribosomal protein S12 The trkB primers were chosen from regions outside the tyrosine kinase domain to avoid recognition by other members of the tyrosine kinase family of genes, particularly the closely related trk and trkC [14]. The primers used to amplify the LNGFR mRNA sequences were 5'-AGCCAACCAGACCGTGTG-TG-3' and 5'-TTGCAGCTGTTCCACCTCTT- 3', the trkB primers were 5'-CCGCTAGGATTTGGTGTACT-3' and 5'-CCACTGTCA-TCAGATGAAAT-3', the c-fos primers were 5'- AGCTATCTCCT-GAAGAGGAA-3' and 5'-AGGCTCCCAGTCTGCTGCAT-3' and the ribosomal protein S12 primers were 5'-GGAAGGCATTGCTG-CTGG-3' and 5'-CTTCAATGACATCCTTGG-3'. The sizes of the amplified products were 663 base pairs (bp) for LNGFR, 664 bp for trkB, 522 bp for c-fos and 368 bp for S12.

3. RESULTS

3.1. *Expression of LNGFR and trkB mRNA in SH-SY5Y cells*

We have found that unstimulated SH-SY5Y cells produce LNGFR and trkB transcripts. As shown in Fig. 1A, PCR amplification with primers specific for LNGFR or trkB gave products of the expected size for the LNGFR (663 bp) and the trkB mRNA (664 bp), respectively. The identity of the trkB PCR product was confirmed both by digestion with appropriate restriction enzymes (*Pst*I and *Hin*dIII) and by hybridization with a trkB-specific probe. An identical pattern of restriction fragments was obtained for the trkB amplification product from rat neocortex, a brain region known to express trkB [20], and from SH-SY5Y cells (Fig. 1B). This finding indicates that the trkB transcripts found in SH-SY5Y cells are similar to those transcribed in vivo.

3.2. *Effect of BDNF on c-fos induction*

The trkB primers used in our experiments are located in the extracellular domain of the trkB gene and therefore do not distinguish between the two classes of trkB transcripts that encode identical extracellular and transmembrane domains, but differ in the intracellular domain by the presence or absence of the catalytic tyrosine kinase region [29]. To investigate whether the LNGFR/trkB transcripts in SH-SY5Y cells encode functional BDNF receptors, we have analyzed the induction of the immediate-early gene c-fos expression as an indicator of responsiveness to BDNF. Fig. 2 shows that c-fos expression was induced two-fold in BDNF-stimulated SH-SY5Y cells as compared to untreated cells, indicating that SH-SY5Y cells express receptors capable of transducing a BDNF signal.

3.3. *Effect of retinoids on LNGFR and trkB mRNA expression*

To investigate whether retinoids influence the expression of BDNF receptors, SH-SY5Y cells were exposed to vitamin A, known to promote neuroblastoma differentiation [30]. Treatment of SH-SY5Y cells with vitamin A induced a marked, dose-dependent increase in LNGFR mRNA (Fig. 3A, upper panel and Fig. 3B). Maximal induction (3-fold) occurred at a concentration of 10 μM. In contrast to LNGFR, a decrease in trkB transcript levels was observed in vitamin A-treated SH-SY5Y cells (Fig. 3A, middle panel and Fig. 3B). The constitutive expression of mRNA encoding ribosomal

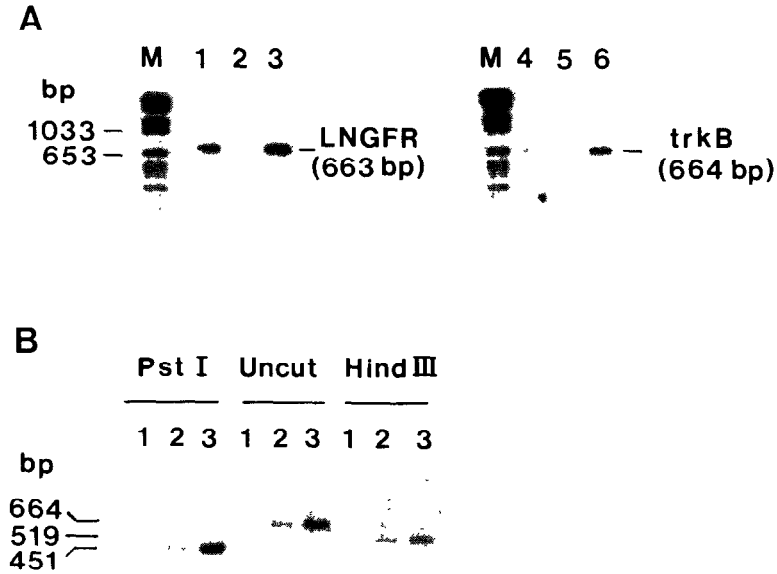

Fig. 1. Detection of LNGFR and trkB transcripts in SH-SY5Y cells. (A) Total RNA isolated from SH-SY5Y cells (lanes 3 and 6) was subjected to RT-PCR amplification using specific primers for LNGFR or for trkB. PCR products were electrophoresed through agarose gels, blotted onto nylon membranes and probed with digoxigenin-labeled DNA probes Total RNA from PC12 cells (lane 1) or from rat neocortex (lane 4) were used as positive controls for LNGFR and trkB amplification, respectively. Lanes 2 and 5, control RT-PCR without RNA. Lanes M, digoxigenin-labeled DNA molecular size markers VI from Boehringer Mannheim. (B) Restriction enzyme analysis of PCR-generated trkB products. Samples were amplified by PCR using trkB primers and treated with restriction enzymes *Pst*I, which produces fragments of 451, 127 and 86 bp, and *Hind*III, which produces fragments of 519 and 145 bp. Restriction fragments were fractionated by electrophoresis on a 1.3% agarose gel and analyzed by Southern blotting. Restriction fragments smaller than 200 bp could only be detected after overexposure and therefore are not seen on the blot. Lanes 1, negative control without RNA; lanes 2, positive control, rat neocortex; lanes 3, unstimulated SH-SY5Y cells.

protein S12 observed in all samples (Fig. 3A, lower panel) confirmed that differences in mRNA levels were due to a specific regulation of the LNGFR and trkB genes. Moreover, exposure of SH-SY5Y cells to derivatives of vitamin A, such as tretinoin, isotretinoin and acitretin, also induced an up-regulation of LNGFR mRNA (Fig. 4A). As demonstrated in Fig. 4B, these retinoids significantly down-regulated trkB mRNA levels, in line with the results obtained with vitamin A. At 10 μM, the retinoid tretinoin elicited the strongest response, followed by vitamin A, isotretinoin and to a much lesser extent acitretin.

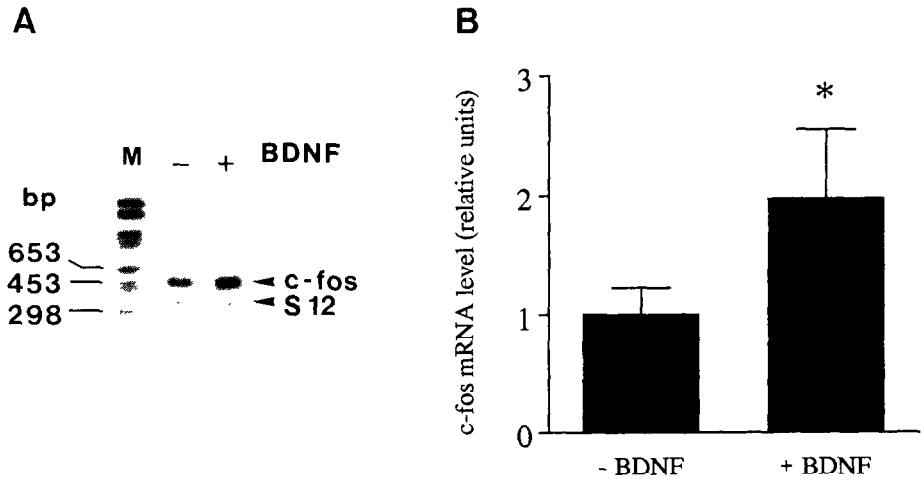

Fig. 2. BDNF induces c-fos transcription in SH-SY5Y cells. Cells were incubated for 60 min either in the absence or presence of BDNF (100 ng/ml) Total RNA was isolated, reverse-transcribed and coamplified for c-fos and control S12 by PCR. (A) Southern blot analysis of PCR products after 21 cycles of amplification. Lane M is as in Fig. 1. (B) Quantitation of c-fos transcripts. Values are the ratio of densitometric scores for c-fos and S12 PCR products. Results are means ± S.E.M. of two independent determinations, each done in triplicate. Statistical analysis performed with Student's *t*-test showed significant differences (*$P < 0.02$) between medium alone and medium plus BDNF.

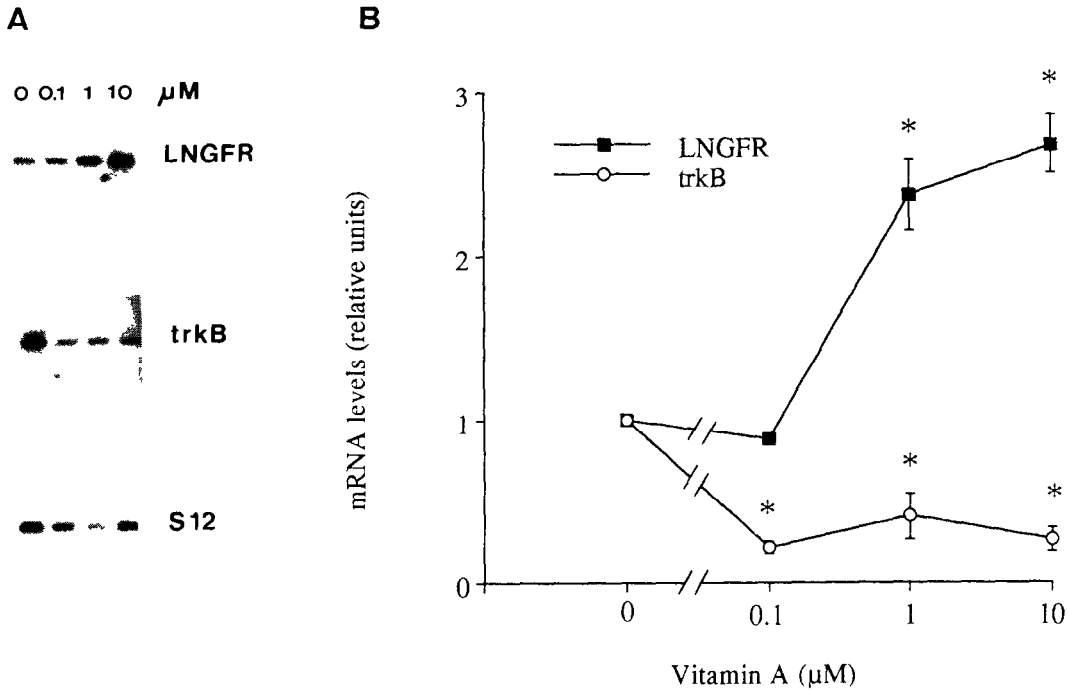

Fig. 3. Effects of vitamin A on the expression of LNGFR and trkB mRNA in SH-SY5Y cells Cells were cultured for 72 h in RPMI 1640/1% FCS in the presence of the indicated concentrations of vitamin A. Total RNA (1 μg) was isolated and subjected to RT-PCR as described in Fig. 1 (A) Southern blot analysis of LNGFR PCR products after 27 cycles of amplification (upper panel), of trkB after 25 cycles of amplification (middle panel) and of control ribosomal protein S12 after 24 cycles of amplification (lower panel) (B) Quantitation of transcripts Values are the ratio of densitometric scores for LNGFR or trkB and S12 PCR products Results are means ± S E.M of three independent determinations, each done in duplicate (*$P < 0.002$)

3.4. *Effect of dbcAMP on LNGFR and trkB levels*

Treatment of SH-SY5Y neuroblastoma cells with dbcAMP promoted morphologic differentiation (Fig. 5) and induced accumulation of LNGFR mRNA. As measured by RT-PCR, LNGFR mRNA levels in treated SH-SY5Y cells were elevated 3-fold compared with controls (Fig. 6). In contrast, trkB mRNA levels were significantly reduced by treatment with dbcAMP (Fig. 6).

4. DISCUSSION

In this report, we present evidence that transcripts encoding LNGFR and trkB are expressed in SH-SY5Y

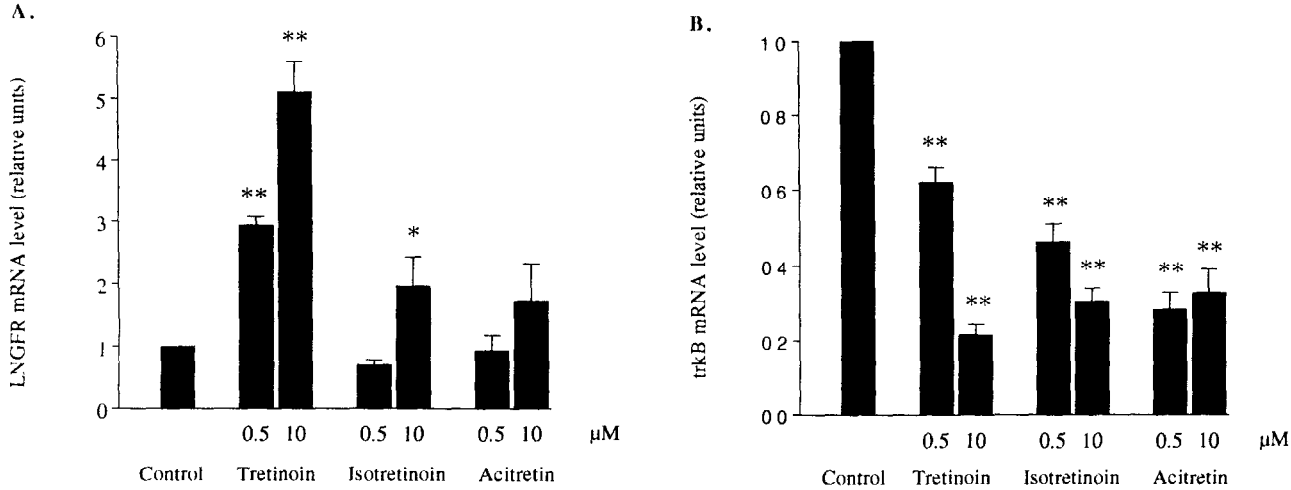

Fig. 4. Effects of vitamin A derivatives on the expression of LNGFR (A) and trkB (B) mRNA in SH-SY5Y cells Cells were cultured for 72 h in the presence of the indicated concentrations of tretinoin, isotretinoin and acitretin. Total RNA was processed for RT-PCR as described in Fig. 1. Results are means ± S E.M. of three independent determinations, each done in duplicate (**$P < 0.002$; *$P < 0.02$).

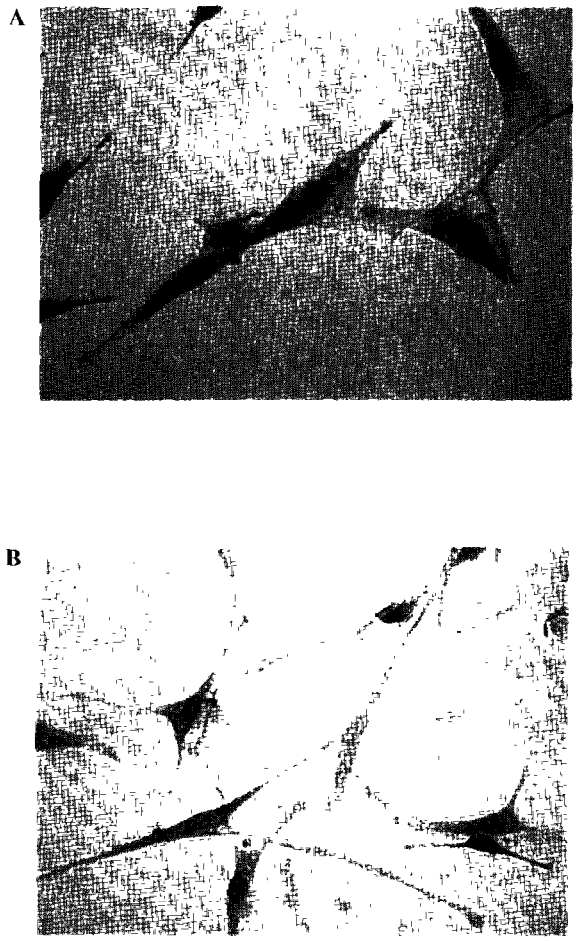

Fig. 5. Morphology of the human SH-SY5Y neuroblastoma cells. Cells were plated as described in Materials and Methods and stained according to May-Grünwald and Giemsa. (A) Undifferentiated SH-SY5Y cells. (B) Differentiated SH-SY5Y cells after 5-day treatment with dbcAMP (1 mM) Magnification × 200

neuroblastoma cells. We demonstrate that SH-SY5Y cells synthesize functional BDNF receptor since interaction of BDNF with SH-SY5Y cells increased the expression of the c-fos gene, whose rapid and transient transcriptional activation is considered to be a marker for BDNF signal transduction. To understand the regulation of BDNF receptor expression better, SH-SY5Y cells were exposed to agents, known to shift neuroblastoma cells to different differentiation state [30]. Our finding that retinoids and cAMP analogs up-regulated LNGFR mRNA, but down-regulated trkB mRNA in SH-SY5Y cells, points to a significant role of LNGFR in differentiated cells. SH-SY5Y cells may thus be used as model system to study the functional importance of the LNGFR.

Expression of functional BDNF receptors on SH-SY5Y cells is consistent with data showing that BNDF can protect SH-SY5Y cells against neurotoxins for dopaminergic neurons [21]. Our finding that BDNF-responsive SH-SY5Y cells express trkB transcripts is in contrast to previous reports which failed to detect trkB in these cells [15,18]. This discrepancy might be explained by the use of the less sensitive Northern blot analysis rather than RT-PCR methodology, or by heterogeneity in SH-SY5Y clones, or differences in culture conditions. In the present study, we demonstrate that retinoids and cAMP analogs up-regulate LNGFR expression in SH-SY5Y cells. Retinoic acid has consistently been shown to increase the expression of LNGFR in other cell types, including LA-N-1 neuroblastoma cells [31], PC12 cells [32] and Sertoli cell line TM4 [33]. Data on the regulation of LNGFR by cAMP are more controversial. While it has been reported that cAMP down-regulated axotomy-induced LNGFR expression of Schwann cells [34], other studies [35] have described a cAMP-independent regulation of LNGFR in the same cells. Further investigations will be required to determine the precise role of adenylate cyclase in the regulation of the LNGFR gene expression.

Our finding that retinoids and cAMP analogs induced down-regulation of trkB expression in SH-SY5Y cells could reflect a decrease in the sensitivity of differentiated SH-SY5Y cells to BDNF. Although the role of the LNGFR is still not fully understood, it is possible that in some cells it serves as a common component for more than one functional neurotrophin receptor. Thus, differentiated SH-SY5Y cells, in which trkB expression is reduced, might still contain trk and trkC, the essential components for binding and responding to other members of the NGF family of neutrophins. It will be of interest to examine whether trk and trkC, the functional receptors for NGF and neurotrophin-3 respectively, are expressed in differentiated SH-SY5Y cells. It is also possible that LNGFR may act in a pathway not involving trk tyrosine kinases.

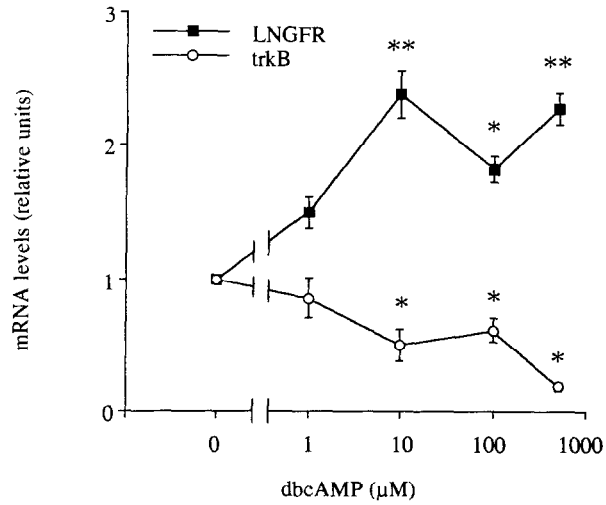

Fig 6. Effects of dbcAMP on the expression of LNGFR and trkB mRNA in SH-SY5Y cells. Cells were cultured for 72 h in the presence of the indicated concentrations of dbcAMP. Samples were processed for RT-PCR as described in Fig 1. Results are means ± S.E.M. of three independent determinations, each done in duplicate (**$P < 0.002$; *$P < 0.02$).

Our results suggest that neuronal differentiation includes modulation of the expression of cell surface neurotrophin receptors. Consistent with this hypothesis, basic fibroblast growth factor was found to initiate neuronal differentiation of a sympathoadrenal progenitor cell line and to induce expression of LNGFR [36].

In the present study, we have shown that the SH-SY5Y neuroblastoma cell line is a suitable model for the study of the regulation of BDNF receptors. This cell line now provides an opportunity for manipulation and analysis of the BDNF receptor signaling pathway.

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