

## Expression of *trkB* in Human Neuroblastoma in Relation to *MYCN* Expression and Retinoic Acid Treatment

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**SUMMARY:** Expression of full-length *trkB* can be found in some highly malignant neuroblastoma tumors with an amplified *MYCN* gene. This contrasts sympathetic neuroblasts, from which neuroblastomas are thought to arise, which neither express *trkB* nor are dependent on the p145<sup>*trkB*</sup> ligands, brain-derived neurotrophic factor (BDNF) or neurotrophin-4/5, for their normal development. In this study we show that *trkB* was expressed in two out of five neuroblastoma tumors with amplified *MYCN*, while no *trkB* expression was observed when the *MYCN* gene was overexpressed in a non-*MYCN*-amplified neuroblastoma cell line. This shows that *MYCN* overexpression per se is not sufficient to induce *trkB* expression. *trkB* expression and BDNF responsiveness in neuroblastoma cells can be induced by all-*trans*-retinoic acid (RA). When SH-SY5Y cells were stimulated with a combination of RA and BDNF, norepinephrine and tyrosine hydroxylase levels were unaltered, showing that the cells did not change toward a more catecholaminergic sympathetic phenotype. However, expression of growth-associated protein 43, indicative of a neuronal phenotype, was elevated. Vesicular acetylcholine transporter, choline acetyl transferase, and neuropeptide tyrosine mRNA levels also increased in RA-BDNF-treated cells, which could suggest that these cells develop into a sympathetic cholinergic phenotype. In addition, treatment with RA-induced expression of the *platelet-derived growth factor receptor- $\alpha$* . As previously shown for BDNF, platelet-derived growth factor stimulated growth of the RA-treated cells, findings that could have clinical relevance. If these receptors mediate a mitogenic signal in vivo also, this might limit the effect of RA treatment on neuroblastoma patients. (*Lab Invest* 2003, 83:813–823).

The childhood malignancy neuroblastoma is derived from cells of the developing sympathetic nervous system. The neuroblastoma cells are arrested at immature stages of sympathetic differentiation, although the stage of tumor cell maturation varies from one tumor to another and often also within a single tumor. This immaturity is frequently manifested in phenotypic characteristics resembling those of sympathetic progenitor cells such as expression of the embryonic and fetal transcription factors dHAND and human achaete-scute homolog-1 (HASH-1) (Gestblom et al, 1997, 1999; Hoehner et al, 1996a; Pålman and Hedborg, 2000). Such progenitor cells give rise to

three major closely related, sympathetic cell lineages: the neuronal/ganglionic, the small intensely fluorescent, and the chromaffin cell lineages (Patterson, 1990). The hormones and growth factors determining rat sympathetic progenitor cell lineage selection are grossly understood and involve glucocorticoids, classical growth factors, and neurotrophins (Anderson, 1993, 1997). For example, development into and along the neuronal lineage requires sequential exposure to insulin-like growth factors, basic fibroblast growth factor, and the neurotrophins nerve growth factor (NGF) and neurotrophin(NT)-3 (DiCicco-Bloom and Black, 1988; Stemple et al, 1988; Verdi and Anderson, 1994; Zackenfels et al, 1995).

In mammals, the neurotrophin family of growth factors consists of NGF, brain-derived neurotrophic factor (BDNF), NT-3, and NT-4 (also known as NT-5) (Barbacid, 1995; Barde, 1994). Their high affinity receptors belong to the *trk* gene family, encoding tyrosine kinase receptors. Thus, NGF binds p140<sup>*trkA*</sup>, BDNF, and NT-4 bind p145<sup>*trkB*</sup>; and NT-3 recognizes p145<sup>*trkC*</sup>. In addition, there is some cross-talk between the various neurotrophins and their receptors (Barbacid, 1995). Both NT-3 and NGF are involved in the

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late phases of sympathetic neuronal differentiation, and in agreement with this, *trkA* and *trkC* are expressed in human sympathetic neuroblasts (Hoehner et al, 1995). Furthermore, mice lacking either *NGF*, *trkA*, or *NT-3* have a reduced number of sympathetic ganglion cells; the *NT-3* effect is apparently being mediated via p140<sup>trkA</sup>, showing explicitly the vital roles of *NGF* and *NT-3* for development and survival of these neurons (Barbacid, 1995). In contrast, expression data indicate that *trkB* is not expressed in human sympathetic neurons or neuroblasts (Hoehner et al, 1995); in *trkB* or *BDNF/NT-4*-deficient mice, the number of sympathetic neurons appears to be unaffected (Barbacid, 1995). This strongly suggests that neither *BDNF* nor *NT-4* have major roles during normal sympathetic neuronal differentiation. However, *trkB* is expressed in the chromaffin cells of human paraganglia at fetal stages, suggesting that p145<sup>trkB</sup> signaling is involved in the development of this sympathetic cell type (Hoehner et al, 1995).

Despite their sympathetic characteristics, neuroblastoma cells can express *trkB*. Truncated *trkB* mRNA is found in some differentiated tumors, while full-length *trkB* mRNA is almost exclusively expressed in *MYCN*-amplified, highly malignant neuroblastoma tumors (Nakagawara et al, 1994). Furthermore, all-*trans*-retinoic acid (RA) induces *trkB* expression in cultured human SH-SY5Y neuroblastoma cells (Kaplan et al, 1993; Matsumoto et al, 1995). The receptors are functional and mediate *BDNF*-induced morphologic differentiation. Under serum-free growth conditions, the RA-*BDNF*-treated cells appear to terminally differentiate (Encinas et al, 2000). These findings are difficult to explain in a context of normal sympathetic neuronal differentiation, considering the apparent lack of *trkB* expression in nonmalignant sympathetic neuroblasts and neurons. One putative explanation could be that the RA-*BDNF*-treated neuroblastoma cells shift toward a nonsympathetic neuronal or to a sympathetic neuroendocrine (chromaffin) phenotype. Previous studies have shown that RA alone induces morphologic differentiation of SH-SY5Y neuroblastoma cells and induces a small increase in choline acetyltransferase activity (Adem et al, 1987; Pålman et al, 1984), without promoting extensive norepinephrine or epinephrine production, the major transmitters of the sympathetic nervous system. However, the SH-SY5Y cells have the capacity to acquire a more developed noradrenergic phenotype, eg, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) induces sympathetic neuronal differentiation in these cells, including a drastic increase in synthesized and stored norepinephrine (Pålman et al, 1981, 1991). Thus, RA might induce a switch in the differentiation program of the SH-SY5Y cells, which would be in keeping with the well-established capacity of RA to shift cells into specific lineages during normal development.

In this report we have investigated if *MYCN* overexpression is causally linked to *trkB* expression in neuroblastoma cells and found that this is not the case. We have also induced *trkB* expression by treating SH-SY5Y cells with RA and analyzed the differentiated

phenotype of RA-*BDNF*-treated SH-SY5Y cells. This treatment led to an increased expression of general neuronal marker genes and the cholinergic marker genes *vesicular acetylcholine transporter (VAChT)* and *choline acetyl transferase (ChAT)*, suggesting an RA-*BDNF*-induced phenotypic shift from a sympathetic noradrenergic toward a cholinergic neuronal phenotype of these neuroblastoma cells. We further show that RA induces expression of *platelet-derived growth factor receptor- $\alpha$  (PDGFR- $\alpha$ )* and that RA-induced growth inhibition could be reversed by stimulation with cognate ligands.

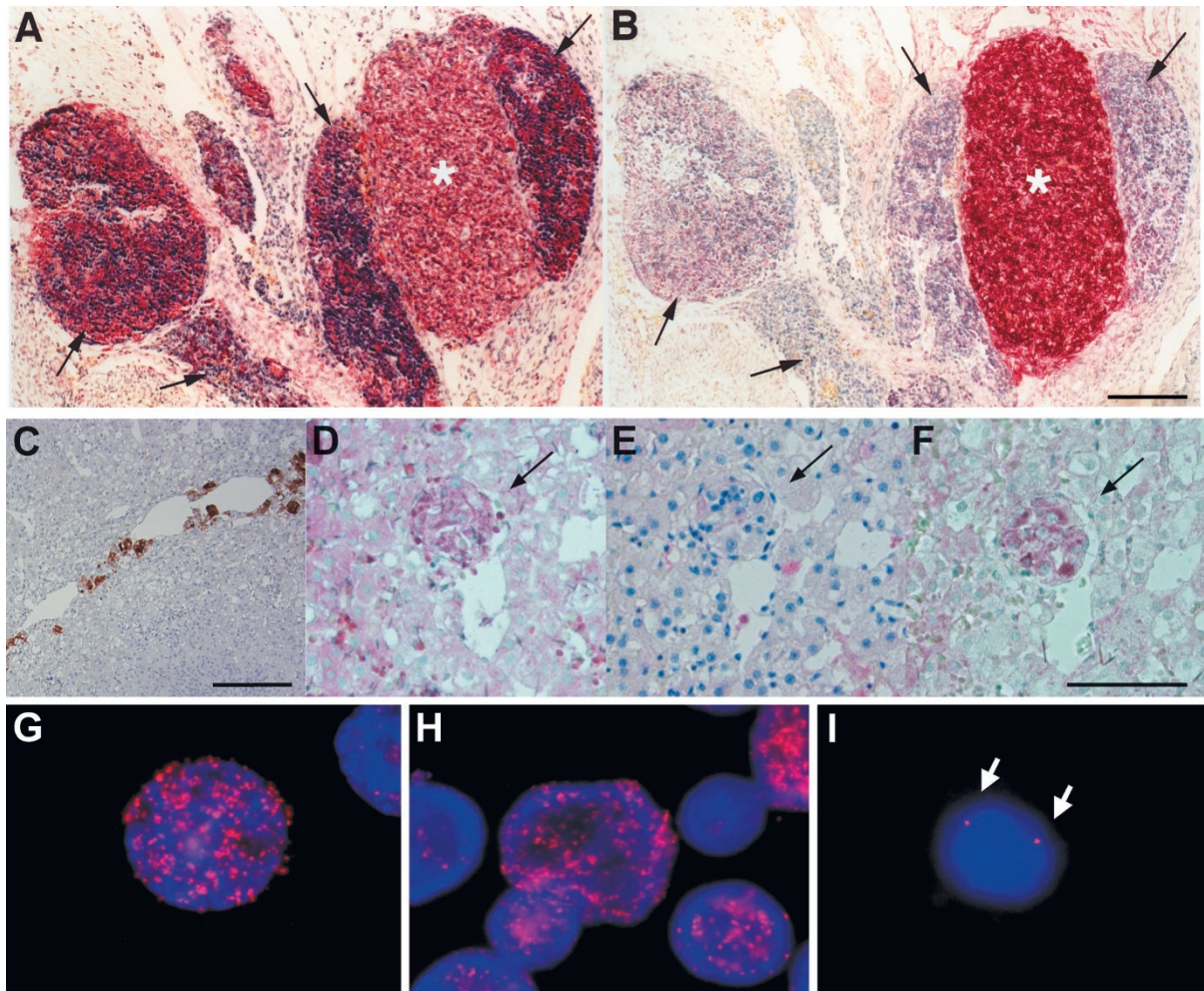
## Results

### *trkB* Expression During Human Fetal Sympathetic Development and in *MYCN*-Amplified Neuroblastoma Tumors

The *trkA* and *trkB* expression in human fetal abdominal cross-sections (developmental weeks 3 to 24) containing sympathetic ganglia and paraganglia was analyzed immunohistochemically. p145<sup>trkB</sup> immunoreactivity was detected solely in sympathetic paraganglia (Fig. 1B), most prominently at the later gestational ages tested (21 to 24 weeks). For comparison, p140<sup>trkA</sup> immunoreactivity was analyzed in serial sections. Expression of p140<sup>trkA</sup> was detected in both sympathetic ganglia and paraganglia (Fig. 1A). The chromaffin cells of the human fetal adrenal medulla, detected by tyrosine hydroxylase expression (Fig. 1C), showed a weak p140<sup>trkA</sup> and a strong p145<sup>trkC</sup> staining, whereas only a trace amount of p145<sup>trkB</sup> immunoreactivity was detected, as exemplified in sections from a 24-week-old fetus (Fig. 1, D to F). In addition, adrenal sympathetic neuroblasts were p140<sup>trkA</sup> and p145<sup>trkC</sup> positive, and p145<sup>trkB</sup> negative (not shown, Hoehner et al, 1995). Thus, during the developmental stages investigated, p145<sup>trkB</sup> immunoreactivity was not detectable in sympathetic neuroblasts, while expression was strong in chromaffin cells of sympathetic paraganglia and weak in adrenal chromaffin cells.

In light of the *trkB* expression pattern in developing sympathetic cells, the expression of *trkB* in highly malignant neuroblastoma cells seen both *in vitro* and *in vivo* (Kaplan et al, 1993; Nakagawara et al, 1994) is difficult to explain because these tumors are considered to be of sympathetic neuronal origin (Hoehner et al, 1996a). Expression of functional, full-length *trkB* in neuroblastoma tumor specimens was only detected in tumors with an amplified *MYCN* gene (Nakagawara et al, 1994), and presumably these tumor cells have a high *MYCN* expression. To verify that high *MYCN* expression due to *MYCN* amplification frequently is seen together with *trkB* expression and that *trkB* expression is not abundant in non-*MYCN*-amplified neuroblastomas, a set of cDNAs derived from tumor specimens was analyzed by semiquantitative PCR for the expression of *MYCN* and full-length *trkB*. As shown in Figure 2, A and B, none of the seven tumors lacking an amplified *MYCN* gene showed detectable *trkB* expression using RA-treated SH-SY5Y cells as a





**Figure 1.**

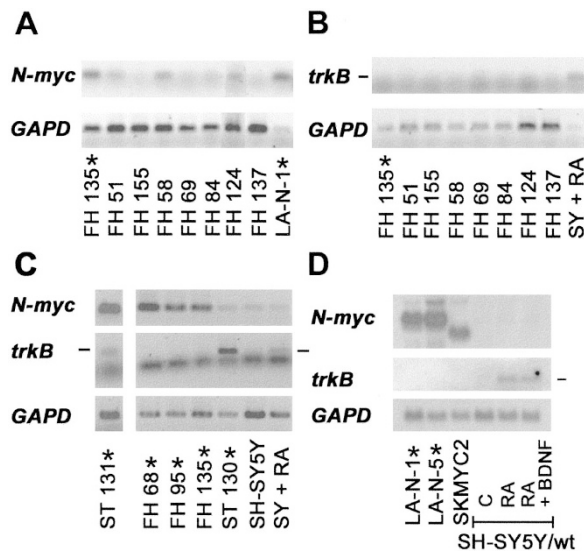
Immunohistochemical characterization of p140<sup>trkA</sup> (A and D), p145<sup>trkB</sup> (B and E), p145<sup>trkC</sup> (F), and tyrosine hydroxylase (TH, C) expression in human fetal sympathetic ganglia, paraganglia, and adrenal chromaffin cells; and *MYCN* amplification in two neuroblastoma tumors analyzed by fluorescence in situ hybridization (G to I). Abdominal cross-sections of a 16-week developmental age fetus were immunostained for p140<sup>trkA</sup> (A) and p145<sup>trkB</sup> (B), respectively, and counterstained with hematoxylin. The panels show sympathetic ganglia (arrows), immunoreactive for p140<sup>trkA</sup> but not p145<sup>trkB</sup>, juxtaposed to a p140<sup>trkA</sup> and p145<sup>trkB</sup> positive sympathetic paraganglion (asterisk). Scale bar, 200  $\mu$ m. (C to F) Chromaffin cells (arrows) of the adrenal medulla of a 24-week-old human fetus stained for TH (C), p140<sup>trkA</sup> (D), p145<sup>trkB</sup> (E), and p145<sup>trkC</sup> (F). Scale bars, 200  $\mu$ m (C) and 100  $\mu$ m (D to F). As seen in D and F, the antibodies directed against p140<sup>trkA</sup> and p145<sup>trkC</sup>, respectively, gave a slight background staining. (G and H) Cells from *MYCN*-amplified cases ST130 and ST131, with high number of *MYCN* gene copies, shown as several red signals in the nucleus. (I) Control cells showing the normal two copies of *MYCN* (arrows).

positive *trkB* control. In contrast, two out of five analyzed tumors with an amplified *MYCN* gene, determined by either Southern blot (Hedborg et al, 1992) or fluorescence in situ hybridization (FISH) analyses (Fig. 1, G and H), expressed *trkB* (Fig. 2C), thus confirming previously reported findings (Nakagawara et al, 1994). To test if overexpression of *MYCN* in non-*MYCN*-amplified neuroblastoma cells is sufficient to induce *trkB* expression, an *MYCN*-transfected SK-N-SH cell clone (SKMYC2) and *MYCN*-amplified neuroblastoma cell lines with high *MYCN* expression were analyzed for *trkB* expression. As shown in Figure 2D, overexpression of *MYCN* did not induce *trkB* expression in SK-N-SH cells, despite the fact that *trkB* expression can be induced by RA in the SK-N-SH derived sub clone, SH-SY5Y (Kaplan et al, 1993). In addition, RA did not induce *MYCN* expression in the SH-SY5Y cells (Fig. 2D). Furthermore, *trkB* was not expressed in any

of the tested cell lines with *MYCN* amplification (Fig. 2D). The cell line data are in accordance with the findings of Nakagawara et al (Nakagawara et al, 1994), who reported only one out of six tested *MYCN* amplified cell lines as *trkB*-positive.

#### Phenotypic Characterization of RA- and RA-BDNF-treated SH-SY5Y Neuroblastoma Cells

Previously we reported that RA induces morphologic (neurite growth) and biochemical (neuron-specific enolase) differentiation of SH-SY5Y cells, but RA treatment inhibited the phorbol ester-induced and differentiation-related increase in norepinephrine levels (Påhlman et al, 1984). At a concentration of 1  $\mu$ M, RA induced a distinct morphologic differentiation of SH-SY5Y cells characterized by growth cone-terminated processes bearing varicosities (Fig. 3C). As

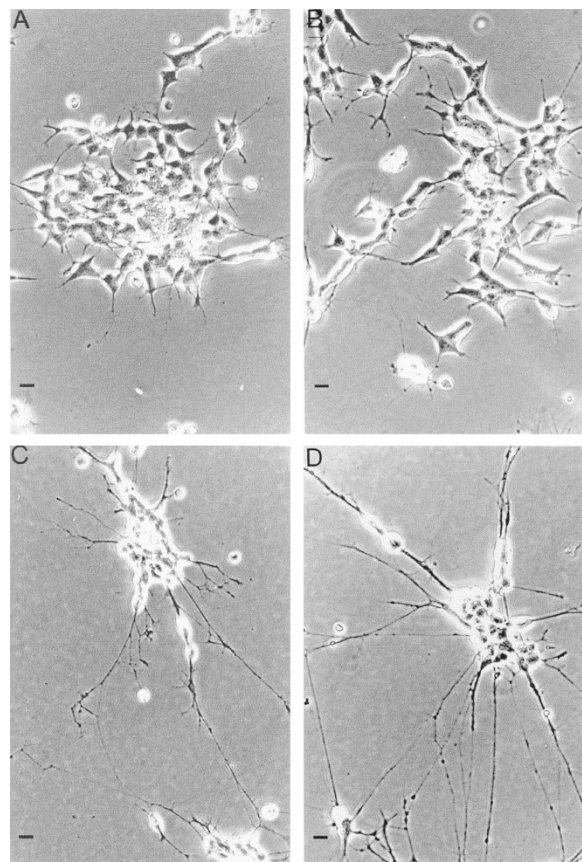


**Figure 2.**

Expression of *MYCN* and *trkB* in neuroblastoma tumors and cell lines. (A) RT-PCR analysis with *MYCN*-specific PCR primers of tumor samples from one *MYCN*-amplified (FH135, marked by an asterisk) and seven nonamplified tumors. After 24 PCR cycles, samples were analyzed by agarose gel electrophoresis. In parallel the same quantity of cDNA was amplified for 22 cycles with *GAPD*-specific primers as a reference for the amount of cDNA in each sample. cDNA from the *MYCN*-amplified LA-N-1 tumor cell line (asterisk) was included as positive control. (B) RT-PCR analysis with *trkB*-specific PCR primers of the same tumor samples analyzed in A with SH-SY5Y/wt cells treated for 6 days with 1  $\mu\text{M}$  RA as a positive control for *trkB* expression. After 36 PCR cycles, samples were analyzed. As in A, amplification with *GAPD*-specific primers for 22 cycles was used as a reference for sample cDNA content. (C) RT-PCR analysis with *MYCN*- and *trkB*-specific PCR primers of tumor samples from five *MYCN*-amplified tumors (asterisks) with cDNA from SH-SY5Y/wt cells ( $\pm$  1  $\mu\text{M}$  RA) included as a negative and positive control, respectively, for *trkB* expression. After 26 (*MYCN*) or 34 (*trkB*) PCR cycles, samples were analyzed. In parallel the same quantity of cDNA was amplified for 23 cycles (for ST131, 24 cycles) with *GAPD*-specific primers as a reference for the amount of cDNA in each sample. (D) Northern blot analysis of expression of *MYCN* (endogenous *MYCN* 2.7 kb, exogenous *MYCN* 2.2 kb) and *trkB* (8.2 kb). SH-SY5Y/wt cells were grown for 6 days in serum-containing medium supplemented with 1  $\mu\text{M}$  RA or 1  $\mu\text{M}$  RA combined with 100 ng/ml brain-derived neurotrophic factor. Fifteen  $\mu\text{g}$  of total RNA was loaded in each lane and *GAPD* (1.5 kb) mRNA levels were used as a reference for the amount of loaded RNA. Asterisks denote *MYCN*-amplified cell lines. The bars in B through D indicate the *trkB* PCR product and mRNA, respectively.

reported by Kaplan et al (Kaplan et al, 1993), BDNF alone (up to 100 ng/ml) did not induce any apparent morphologic changes compared with nontreated controls (Fig. 3, A and B), while RA treatment led to *trkB* expression and BDNF responsiveness. Compared with the morphology of cells treated with RA only, RA-BDNF-treated cells had a more distinct phenotype, manifested by longer neurites and a considerably higher number of varicosities (Fig. 3, C and D).

As the phenotype of the RA-BDNF-treated cells grown in serum has not been further characterized and the induced *trkB* expression suggested that these cells did not develop into a noradrenergic, neuronal sympathetic phenotype, we extended the characterization by employing marker genes previously used to phenotypically describe the neuronal and neuroendocrine cells of the developing human sympathetic nervous system (Gestblom et al, 1997, 1999; Hedborg et al, 1995; Hoehner et al, 1996a). The axonal nature of the



**Figure 3.**

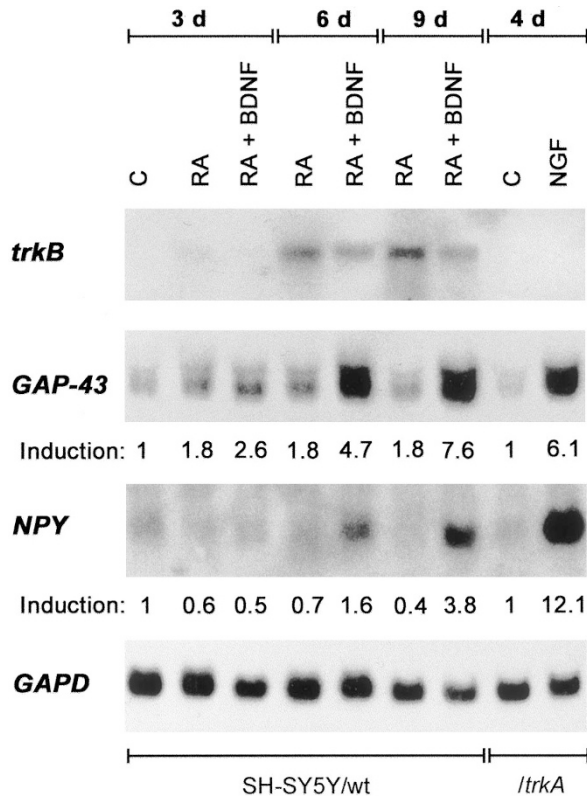
Morphology of wild-type SH-SY5Y cells cultured in serum-containing medium for 6 days with the following additives: (A) nontreated controls, (B) 100 ng/ml brain-derived neurotrophic factor (BDNF), (C) 1  $\mu\text{M}$  RA, and (D) 1  $\mu\text{M}$  RA + 100 ng/ml BDNF. Bars represent 25  $\mu\text{m}$ .

neurites of both RA- and RA-BDNF-treated cells was corroborated by the increased expression of *GAP-43* (Fig. 4), a gene encoding an axonal protein involved in growth cone motility and synapse formation. RA induced less than a 2-fold increase in *GAP-43* expression for up to 9 days of treatment. Consistent with previously published results from experiments performed under slightly different conditions (Encinas et al, 1999; Lesser et al, 1997), RA-BDNF treatment resulted in a 5- to 8-fold increase after 6 and 9 days of cell exposure. For comparison, we also included in our analysis SH-SY5Y/*trkA* cells treated for 4 days with NGF (Fig. 4), a treatment leading to increased *GAP-43* expression and neuronal sympathetic differentiation (Edsjö et al, 2001; Lavenius et al, 1995).

#### Neurotransmitter Synthesis in RA- and RA-BDNF-Treated SH-SY5Y Cells

Norepinephrine and NPY are two important neurotransmitters produced in catecholaminergic cells of the sympathetic nervous system. The concentration of the major neurotransmitter, norepinephrine, was analyzed in RA- and RA-BDNF-treated cells. No significant increase in norepinephrine levels compared with control cells was detected, but there was a tendency

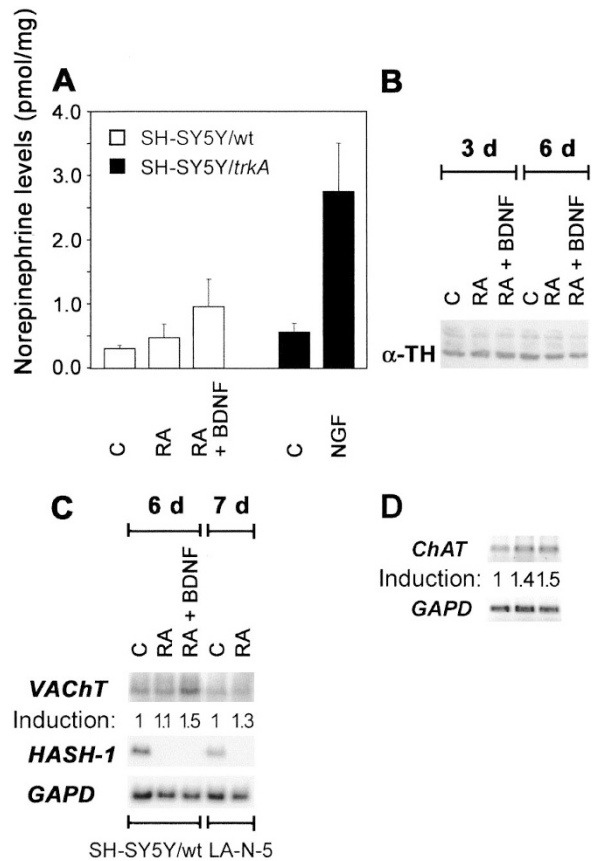




**Figure 4.** Northern blot analysis of *trkB*, *GAP-43*, and *NPY* expression in RA and RA–brain-derived neurotrophic factor (BDNF)–treated SH-SY5Y/wt cells and in nerve growth factor–treated SH-SY5Y/*trkA* cells. Cells were grown in serum-containing medium, with the addition of 1  $\mu$ M RA and 1  $\mu$ M RA + 100 ng/ml BDNF (SH-SY5Y/wt) or 100 ng/ml NGF (SH-SY5Y/*trkA*), for the indicated time periods. Total RNA (15  $\mu$ g) was analyzed for the expression of *trkB* (8.2 kb), *GAP-43* (1.4 kb), and *NPY* (0.8 kb), using *GAPD* (1.5 kb) mRNA levels as a measure of the amount of loaded mRNA. The band intensities were analyzed with phosphorimaging, and the relative amounts of mRNA (normalized to *GAPD* levels and controls) are shown. The increase in *trkB* mRNA levels could not be quantified since no reliable signal was obtained from the control cell RNA.

toward increased norepinephrine levels in the RA-BDNF–treated cells (Fig. 5A). However, if real, this effect was small compared with the 6-fold increase in the norepinephrine concentration induced by NGF treatment of SH-SY5Y/*trkA* cells (Fig. 5A). The rate-limiting enzyme of the catecholamine synthesis, tyrosine hydroxylase (TH), was analyzed in these cells at the protein level by Western blotting. Neither RA nor the combination of RA and BDNF induced an increased TH-expression during a time period (3 to 6 days) when the cells differentiated morphologically and biochemically (Fig. 5B). Instead a slight reduction in the TH levels was observed after 6 days of RA and RA-BDNF treatment.

The expression of *NPY*, which in the sympathetic nervous system characteristically is expressed in the neuronal but not in the chromaffin paraganglionic lineage (Gestblom et al, 1997), increased significantly (approximately 4-fold) after 9 days of RA-BDNF exposure (Fig. 4). The *NPY* mRNA levels were considerably lower after stimulation for shorter time periods, and treatment with RA alone resulted in decreased *NPY* mRNA levels at all analyzed time points (Fig. 4).



**Figure 5.** Phenotypical characterization of RA- and RA–brain-derived neurotrophic factor (BDNF)–treated SH-SY5Y cells. (A) HPLC analysis of norepinephrine levels in cell lysates from SH-SY5Y/wt cells treated for 6 days with 1  $\mu$ M RA or 1  $\mu$ M RA + 100 ng/ml BDNF ( $n = 5$ ) and in SH-SY5Y/*trkA*-transfected cells treated for 4 days with 100 ng/ml NGF ( $n = 7$ ). The mean norepinephrine levels are represented by bars; error bars indicate SEM. (B) Western blot analysis of tyrosine hydroxylase protein levels in RA- and RA-BDNF–treated SH-SY5Y/wt cells. Cells were grown in serum-containing medium, with the addition of 1  $\mu$ M RA and 1  $\mu$ M RA + 100 ng/ml BDNF. After 3 or 6 days, cells were harvested, and 30  $\mu$ g of whole cell lysate proteins was loaded in each lane. (C) Northern blot analysis of *VACHT* and *HASH-1* expression in RA- and RA-BDNF–treated SH-SY5Y/wt and LA-N-5 cells. Cells were grown in serum-containing medium, with the addition of 1  $\mu$ M RA or 1  $\mu$ M RA + 100 ng/ml BDNF, where indicated. After 6 (SH-SY5Y/wt) and 7 days (LA-N-5), cells were harvested and lysed. Fifteen  $\mu$ g of total RNA from each sample was analyzed for the expression of *VACHT* (2.5 kb) and *HASH-1* (2.7 kb), using *GAPD* (1.5 kb) mRNA levels as a measure of the amount of loaded mRNA. The band intensities were analyzed with densitometry, and the relative amounts of mRNA (normalized to *GAPD* levels and controls) are shown. (D) RT-PCR analysis of samples from SH-SY5Y/wt cells grown as in (C, RA, RA + BDNF), using *ChAT*-specific primers. After 32 PCR cycles, samples were analyzed. In parallel the same quantity of cDNA was amplified for 19 cycles using *GAPD*-specific primers and was used as a reference for sample cDNA content.

Although the elevation of *NPY* expression in cells treated for 9 days with RA-BDNF was significant, it was lower than that seen in TPA-treated SH-SY5Y/wt cells and NGF-treated SH-SY5Y/*trkA* cells (Fig. 4) and in previous results (Lavenius et al, 1995; Pählman et al, 1991).

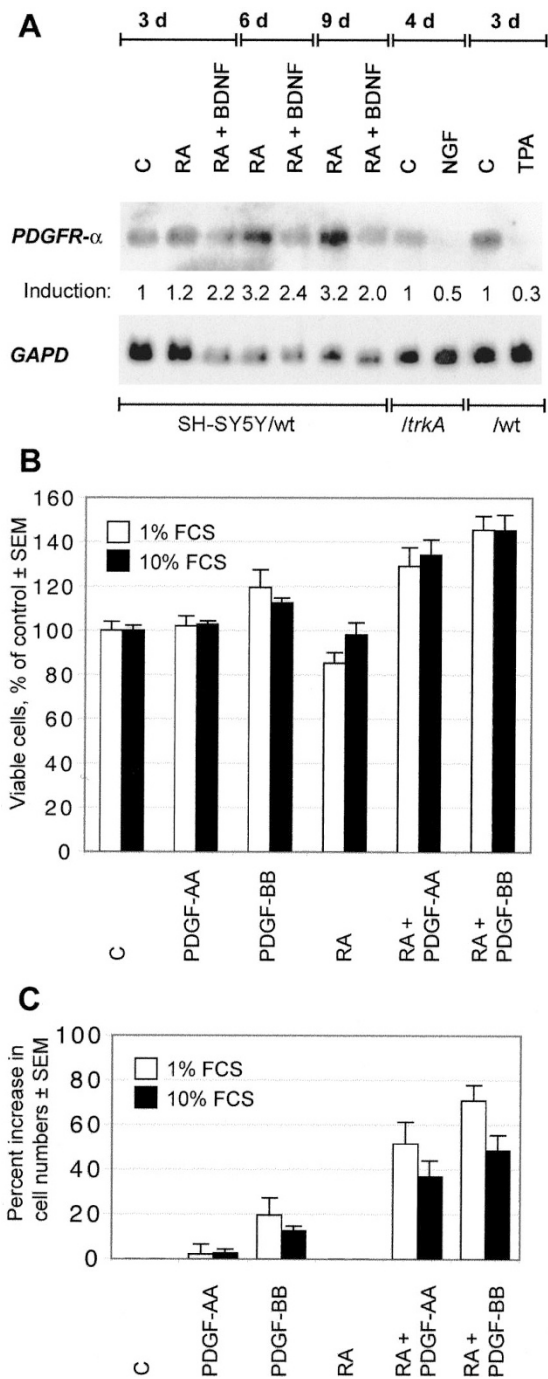
During normal development, a subpopulation of catecholaminergic sympathetic neuroblasts transdifferentiate and become cholinergic. Neuroblastoma cells, as well as catecholaminergic sympathetic neuroblasts, show cholinergic traits with weak expression

of cholinergic marker genes like *ChAT* (Biedler et al, 1978; Francis and Landis, 1999; Yokomori et al, 1983). We have previously reported that RA treatment of SH-SY5Y cells promotes a cholinergic phenotype measured as an increase in choline acetyltransferase activity (Adem et al, 1987). A switch to a cholinergic phenotype was recently reported to occur during RA treatment of human NB69 and LA-N-5 neuroblastoma cells (Hill and Robertson, 1997; Handler et al, 2000). We, therefore, analyzed the effect of BDNF on RA-treated SH-SY5Y cells on the expression of the cholinergic marker genes, the acetylcholine transporter *VACHT* and *ChAT*. As shown in Figure 5C, RA induced a minute increase in *VACHT* expression, which was enhanced (1.5-fold over control) by BDNF. The latter increase was in parity with that seen in RA-treated LA-N-5 cells (1.3-fold increase) used as a positive control of neuroblastoma cells acquiring a cholinergic phenotype (Hill and Robertson, 1997). As expected, *ChAT* expression was also induced by RA and RA-BDNF (Fig. 5D). The induced cholinergic features of the RA and RA-BDNF-treated SH-SY5Y cells were also accompanied by a complete down-regulation of *HASH-1* (Fig. 5C), a transiently expressed transcription factor essential for the commitment and differentiation of migrating neural crest cells toward a noradrenergic sympathetic neuroblastic phenotype.

#### PDGF Receptor Expression in RA- and RA-BDNF-Treated SH-SY5Y Cells

The RA-treated SH-SY5Y cells expressing *trkB* are highly proliferating in the presence of BDNF and serum, and the BDNF stimulation also enhances a migratory response (Matsumoto et al, 1995). Thus, the BDNF-induced differentiation does not lead to growth inhibition, as opposed to for instance the TPA-induced differentiation of SH-SY5Y cells (Påhlman et al, 1991). The development of the phorbol ester-driven phenotype requires serum or a growth factor such as PDGF. The receptor, *PDGFR-α*, is expressed in SH-SY5Y cells and transduces a mitogenic response upon stimulation with PDGF-AA (Påhlman et al, 1992). As this receptor is down-regulated in TPA-differentiated SH-SY5Y cells, presumably as part of an induced sympathetic differentiation program (Figure 6A; previous results [Påhlman et al, 1992]), we analyzed the expression pattern of this receptor in RA and RA-BDNF-treated cells. RA induced a several-fold increase in *PDGFR-α* expression, and the combination with BDNF slightly reduced that increase. In contrast, both NGF-treated SH-SY5Y/*trkA* and TPA-treated SH-SY5Y wild-type cells down-regulated *PDGFR-α* (Fig. 6A). Thus, with regard to *PDGFR-α* expression, there was a distinct difference between RA-treated and TPA-treated SH-SY5Y cells.

RA induces differentiation accompanied by growth inhibition in many neuroblastoma cell lines, which is the rationale behind treatment of minimal residual disease in neuroblastoma. It was therefore of interest to test whether PDGF-AA (binds exclusively to the *PDGFR-α*) and PDGF-BB (binds both *PDGFR-α* and



**Figure 6.**

Platelet-derived growth factor receptor (*PDGFR*) expression and PDGF effects on cell numbers. (A) Northern blot analysis of *PDGFR-α* expression in SH-SY5Y/wt cells treated with 1  $\mu$ M RA, 1  $\mu$ M RA + 100 ng/ml brain-derived neurotrophic factor and 16 nM TPA, and in SH-SY5Y/*trkA* cells treated with 100 ng/ml NGF. The cells were harvested after the indicated treatment periods, and 15  $\mu$ g of total RNA was analyzed for the expression of *PDGFR-α* (6.5 kb), using *GAPD* (1.5 kb) mRNA levels as a measure of the amount of loaded RNA. The band intensities were analyzed with phosphorimaging, and the relative amounts of mRNA (normalized to *GAPD* levels and controls) are shown. (B) Number of viable cells in SH-SY5Y/wt cell cultures, grown for 3 days in 1% or 10% FCS, as measured by MTT assay ( $n = 8$  to 12). Where indicated, 50 ng/ml PDGF-AA, 50 ng/ml PDGF-BB, and/or 1  $\mu$ M RA was added to the medium. (C) Isolated effect of PDGFs  $\pm$  RA. Data from 6B, expressed as percent increase in cell numbers in cultures treated with PDGF-AA or -BB  $\pm$  RA, compared with the amount of viable cells in untreated control and RA-treated cells, respectively.

PDGFR- $\beta$ ) could mitogenically stimulate the RA-treated SH-SY5Y cells. In the absence of RA, SH-SY5Y cell growth was virtually unaffected by PDGF-AA treatment but increased slightly with PDGF-BB (Fig. 6, B and C). In contrast but in agreement with PDGFR- $\alpha$  expression data, PDGF-AA potentially stimulated cell growth of RA-treated SH-SY5Y cells, both at 1% and 10% serum concentration. PDGF-BB, on the other hand, stimulated growth also in non-RA-treated cells, an effect markedly potentiated in RA-treated cells. Considering the significant effect of PDGF-AA on these cells, the augmented PDGF-BB response was conceivably due to PDGF-BB stimulation of the up-regulated PDGFR- $\alpha$  (Fig. 6, B and C).

## Discussion

Data from characterization of neuroblastoma tumors employing a broad panel of neuronal and neuroendocrine marker genes are consistent with neuroblastomas arising from cells of the sympathetic nervous system (Hoehner et al, 1996a). There are, however, examples of genes expressed in neuroblastoma that usually are not transcribed in developing or mature sympathetic cells. Many of these genes are expressed in other neural crest derived lineages, and one hypothesis is that the expression of such genes reflects the immature or stem cell character of these neuroblastoma cells (Påhlman and Hedborg, 2000). The expression of *trkB* in some tumors and in RA-treated cultured neuroblastoma cells is one example in which the expression profile does not appear to easily fit into such a developmental model. Although *trkB* is expressed in developing chromaffin cells of the paraganglia, the differentiation markers of RA-BDNF-treated SH-SY5Y cells are not in keeping with a paraganglionic phenotype. The formation of long neurites, and the GAP-43 induction in RA-BDNF-treated cells, indicate that this treatment promotes a neuronal phenotype (Gestblom et al, 1997). Furthermore, the low norepinephrine levels and lack of TH induction also argue against these cells becoming chromaffin because high catecholamine production is a hallmark of a chromaffin cell. The phenomenon as such, ie, trans-differentiation of neuroblastoma cells displaying sympathetic neuronal features into a sympathetic extra-adrenal chromaffin lineage, can occur spontaneously, preferentially in low-stage neuroblastoma tumors (Gestblom et al, 1997). Instead, RA-BDNF induced the expression of cholinergic marker genes; the increase in *VAcHT* expression appeared primarily to be a BDNF effect, while the increased *ChAT* expression was mainly RA-induced. These changes, together with the increase in *NPY* expression, might suggest that the RA-BDNF-treated SH-SY5Y cells develop into a sympathetic cholinergic neuronal lineage, although it is to our knowledge not known if human sympathetic cholinergic neuroblasts or neurons express *trkB*.

There are now several examples of cultured neuroblastoma cells that respond to RA treatment by expressing cholinergic features (Handler et al, 2000; Hill

and Robertson, 1997), suggesting that this is a general effect of RA on neuroblastoma cells. Consistent with this conclusion, cultured sympathetic neuroblasts also switch from a catecholaminergic to a cholinergic phenotype when treated with RA (Matsushima and Bogenmann, 1993). As discussed below, the in vitro RA-induced tumor cell phenotype, including expression of functional growth factor receptors, might in vivo result in tumors with new growth properties potentially adding to an increased malignant behavior; however, we do not know if the induced *trkB* and *PDGFR- $\alpha$*  expression is linked to the cholinergic phenotype. To our knowledge, there is only one report in which the clinical behavior of catecholaminergic and cholinergic neuroblastomas has been compared (Yokomori et al, 1983). Based on pretreatment measurements, no clear difference in outcome between catecholaminergic and cholinergic tumors was found in this report. However, in relapsed tumors treated with chemotherapy or radiation, there was a tendency toward a change from a catecholaminergic to a cholinergic phenotype, which might suggest that the cholinergic phenotype is associated with aggressive tumor behavior (Yokomori et al, 1983).

Highly malignant neuroblastomas, including established cell lines, have many characteristics (eg, *HASH-1* and *MYCN* expression) in common with the sympathoadrenal progenitor cell defined primarily in rodents. Neuroblastoma cells might thus be arrested at such an early differentiation stage that they can behave as progenitor cells. If this is indeed the case, it is reasonable to assume that conditions can be found at which cultured neuroblastoma cells can be forced into cell lineages other than those of the sympathetic nervous system. The neural crest-derived progenitor cells giving rise to the sympathetic nervous system are also progenitors for some of the neurons of the enteric nervous system (Blaugrund et al, 1996; Carnahan et al, 1991). Interestingly, enteric neurons do express *trkB* (Hoehner et al, 1996b) and *NPY* (Gaetano et al, 1991; Gordon-Weeks, 1988). Furthermore, although mature intrinsic enteric neurons appear not to express norepinephrine or epinephrine, immature enteric neurons do (Kapur, 1993). Thus, as many of the characteristics of the RA-BDNF-treated cells appear to be similar to those of immature or differentiating enteric neurons, differentiation into this lineage is, formally, an alternative explanation of our data.

Amplification of *MYCN* in neuroblastomas correlates strongly to poor prognosis. *MYCN* functions as a heterodimeric transcription factor, forming a complex with Max. It is generally believed that overexpression of *MYCN* in neuroblastoma results in altered gene expression, although few target genes have been identified (Lasorella et al, 2000; Schwab, 2000; Shohet et al, 2002). One question we wanted to answer was whether high *MYCN* expression was directly linked to the *trkB* expression observed in some neuroblastomas. We conclude that the *trkB* expression detected in neuroblastoma tumors is not directed by high *MYCN* expression. It is a fact, though, that expression of full-length *trkB* in neuroblastoma specimens primar-



ily is found in tumors with *MYCN* amplification. Whether the *trkB* expression is regulated via co-amplified genes like *DDX1* (George et al, 1997; Godbout and Squire, 1993) or merely is a reflection of a phenotypic heterogeneity among neuroblastoma tumors, possibly requiring high *MYCN* expression, needs to be further addressed.

The fact that RA induces *trkB* and *PDGFR- $\alpha$*  expression in SH-SY5Y cells and that these receptors transduce mitogenic signals to these cells are findings that might have clinical relevance. RA and RA homologues have for a long time been recognized as promising drugs for treatment of neuroblastoma, with the anticipation that they will induce tumor cell differentiation and growth arrest in vivo, similar to the effects seen in vitro. With one noticeable and promising recent exception (Matthay et al, 1999), RA and its homologues have not lived up to these expectations, and RA treatment has, so far, not dramatically changed the outcome of neuroblastoma patients. One molecular explanation for a weak in vivo effect of RA on neuroblastoma growth could be that this treatment induces growth factor receptor expression, similar to the in vitro results presented in this study. If that is the case, these growth factor receptors could transduce mitogenic signals, counteracting the growth inhibitory effect that would follow from the RA-induced differentiation. A future treatment strategy might therefore be to combine RA with one or several of the novel receptor tyrosine kinase inhibitors now becoming available.

## Materials and Methods

### Cell Cultures, Tumor Materials, and Assessment of Cell Numbers

SH-SY5Y wild-type (SH-SY5Y/wt) neuroblastoma cells, a neuroblastic subclone of the non-*MYCN*-amplified SK-N-SH cell line (Biedler et al, 1973), as well as stably *trkA*-transfected SH-SY5Y cells (SH-SY5Y/*trkA*, clone 6:2) (Lavenius et al, 1995), were cultured in Eagle's minimum essential medium with the following additives: 10% FCS (FCS), 100 IU/ml penicillin V, and 100  $\mu$ g/ml streptomycin. LA-N-1, LA-N-2, and LA-N-5 cells were grown in RPMI 1640 medium with 10% FCS and the same antibiotics added. To induce differentiation the following additives were used: RA (Sigma, St. Louis, Missouri), human recombinant BDNF (PeproTech EC Ltd., London, United Kingdom), mouse NGF 2.5S (Promega, Madison, Wisconsin), and PDGF-AA and -BB (PeproTech). Media, FCS, and antibiotics were from Life Technologies Inc. (Rockville, Maryland). The SKMYC2 clones of SK-N-SH cells transfected with *MYCN* has previously been described (Judware and Culp, 1995). The RNA analyzed was extracted from cells grown with 200  $\mu$ g/ml of Hygromycin B (Calbiochem, San Diego, California). The amount of viable cells in cell cultures was analyzed measuring the conversion of the tetrazolium salt MTT to formazan (CellTiter 96, Promega). Cells were plated at a density of 7500 cells/well in 96-well plates with factors used added prior to the addition of cells.

The tumor material used has been described previously (Hedborg et al, 1992; Martinsson et al, 1995). *MYCN* amplification was determined by either Southern blot (Hedborg et al, 1992; Martinsson et al, 1995) or FISH analyses (Martinsson et al, 1995). FISH analysis was performed as previously described (Fröstad et al, 1999).

### Northern Blot and Semiquantitative RT-PCR Analyses

The guanidine-isothiocyanate/phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) was used for isolation of total cellular RNA. Fifteen  $\mu$ g of RNA was electrophoretically separated on a 1% agarose-formaldehyde gel and blotted onto a nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Uppsala, Sweden). The following cDNA probes were labeled with [<sup>32</sup>P] dCTP using an oligonucleotide labeling kit (Amersham Pharmacia Biotech): *Neuropeptide tyrosine (NPY)* (Minth et al, 1986), *MYCN* (Kohl et al, 1983), *glyceraldehyde-3-phosphate-dehydrogenase (GAPD)* (Tso et al, 1985), *growth-associated protein 43 (GAP-43)* (Örtoft et al, 1993), *trkA* (Martin-Zanca et al, 1989), *trkB* (Klein et al, 1989), *VACHT* (a 1605 bp probe amplified from LA-N-2 cDNA, using the 30-mer 5'-CGC GGA TCC ATG GAA TCC GCG GAA CCT GCG-3' as forward and the 29-mer 5'-CGC GAA TTC TAG CTG CGG GTG TAG TAG TA-3' as reverse primer), and human *PDGFR- $\alpha$*  (Claesson-Welsh et al, 1989). Hybridizing probes were visualized by autoradiography, and RNA levels were quantified and related to the corresponding *GAPD* mRNA level by phosphor image analysis, using a Fuji BAS 2000 equipment (Fujifilm, Tokyo, Japan) or by densitometric analysis.

Total RNA from snap-frozen tumor specimens and cultured cells were extracted as described above and treated with RNase-free DNase I (Appligene Oncor, Illkirch, France) prior to cDNA synthesis. cDNA synthesis was performed using the Gibco BRL SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies Inc.). Duplicates of each sample were made and the reverse transcriptase was added to one of them (RT+), while the other (RT-) was used as a negative control for contaminating genomic DNA in the samples. The cDNA was purified using the QIAquick PCR Purification kit (Quiagen, Venlo, the Netherlands).

In the subsequent PCR reactions, template cDNAs were mixed with 10 $\times$  PCR buffer (Sigma) and 20 nM of each primer in a total volume of 60  $\mu$ l along with 2.5 U of *Taq*DNA polymerase (Sigma). Amplification was carried out for 19 to 36 cycles, with a denaturation temperature of 95° C, annealing temperature of 57° C, and an extension temperature of 72° C for 30 seconds each. RT- samples were run in parallel for at least as many cycles as the RT+ samples to check for genomic DNA. Ten  $\mu$ l of the collected aliquots were loaded on a 1% agarose gel stained with SYBR Green I Nucleic Acid Gel Stain (Roche, Basel, Switzerland). Band intensities were recorded and analyzed using LAS-1000 CCD imaging equipment with accompany-



ing image analyzing software (Fujifilm). For each primer set, the exponential phase of amplification was determined by removing aliquots at four different cycle numbers. The genes analyzed were *ChAT* (forward primer: 5'-CTGCTGCTATGGTCTGTGG-3', reverse: 5'-TCAAG-GTTGGTGTCCCTGGC-3'); *GAPD* (forward primer: 5'-CCATCACCATCTCCAGGAGC-3', reverse: 5'-AGTGA-TGGCATGGACTGTGGTC-3'); *MYCN* (forward primer: 5'-TTCCTCTCCAACACCAAGG-3', reverse: 5'-CGCCT-CGCTCTTTATCTTCT-3'); and *trkB* (forward primer: 5'-GAG CAT CAT GTA CAG GAA AT-3', reverse: 5'-CTT GAT GTT CTT CCT CAT GT-3'). The *trkB* primers will generate a 235-bp PCR product covering a part of the 3' end-coding for the tyrosine kinase domain, thereby only amplifying *trkB* cDNA generated from full-length transcripts and not the cDNAs from the two known truncated *trkB* mRNAs (Barbacid, 1994; Klein et al, 1989, 1990; Middlemas et al, 1991).

### Immunohistochemical and Western Blot Analyses

Human fetal tissue was obtained from either elective or spontaneous abortions from 9- to 25-weeks gestational age, kindly provided by Dr. B. Sandstedt, Stockholm. Ethical approval (Dnr 93-216) was obtained from the local ethical committee of Karolinska Hospital, Stockholm, Sweden. p140<sup>trkA</sup>, p145<sup>trkB</sup>, and p145<sup>trkC</sup> expression were analyzed immunocytochemically as described (Hoehner et al, 1995), in which the specificities of the antisera employed (Santa Cruz, CA) were characterized and reported. Sections were counter-stained with hematoxylin.

For Western blot analysis, cells were lysed in 10 mM Tris-HCl (pH 7.2), 160 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, 1 mM EDTA in the presence of Complete Protease Inhibitor (Roche). For TH-protein-expression analysis, 30  $\mu$ g of whole-cell lysate protein was separated on an SDS-PAGE gel and blotted onto a Hybond C extra membrane (Amersham Pharmacia Biotech). A monoclonal antityrosine hydroxylase antibody (Roche), diluted 1:500, was used along with a horseradish peroxidase coupled secondary antibody diluted 1:5000 (Amersham Pharmacia Biotech).

### Norepinephrine Analysis

RA and neurotrophin-treated cells ( $10^6/10$ -cm plate) were grown for 4 days and then washed and harvested in ice-cold PBS. The pelleted cells were resuspended in 0.5 M trichloroacetic acid and then centrifuged at 21,000  $\times$ g for 5 minutes in 4° C. The supernatant was saved and pH adjusted to pH 4 with NaOH. The pellet was resuspended in 1 M NaOH for protein determination according to Bradford. After addition of an internal standard (3,4-dihydroxynorepinephrine) the supernatant samples were extracted and derivatized with 1,2 diphenylethylenediamine as previously described (van der Hoorn et al, 1989). The samples were analyzed by HPLC using a 25  $\times$  2-mm precolumn (Perisorb RP 18, 30- to 40- $\mu$ m particle diameter) and a 250  $\times$  4.6-mm column (Hypersil ODS,

5- $\mu$ m particle diameter) using 50 mM Na acetate (pH 7.0):acetonitrile:methanol (5:4:1) as mobile phase. The fluorescence was detected with either a Shimadzu RF-10A XL or a Jasco Fluorescence Detector 821, and the signal was integrated with a Shimadzu C-R3A integrator.

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### References

- Adem A, Mattsson ME, Nordberg A, and Pålman S (1987). Muscarinic receptors in human SH-SY5Y neuroblastoma cell line: Regulation by phorbol ester and retinoic acid-induced differentiation. *Brain Res* 430:235-242.
- Anderson DJ (1993). Molecular control of cell fate in the neural crest: The sympathoadrenal lineage. *Annu Rev Neurosci* 16:129-158.
- Anderson DJ (1997). Cellular and molecular biology of neural crest cell lineage determination. *Trends Genet* 13:276-280.
- Barbacid M (1994). The Trk family of neurotrophin receptors. *J Neurobiol* 25:1386-1403.
- Barbacid M (1995). Neurotrophic factors and their receptors. *Curr Opin Cell Biol* 7:148-155.
- Barde YA (1994). Neurotrophic factors: An evolutionary perspective. *J Neurobiol* 25:1329-1333.
- Biedler JL, Helson L, and Spengler BA (1973). Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Res* 33:2643-2652.
- Biedler JL, Roffler-Tarlov S, Schachner M, and Freedman LS (1978). Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. *Cancer Res* 38:3751-3757.
- Blaugrund E, Pham TD, Tennyson VM, Lo L, Sommer L, Anderson DJ, and Gershon MD (1996). Distinct subpopulations of enteric neuronal progenitors defined by time of development, sympathoadrenal lineage markers and Mash-1-dependence. *Development* 122:309-320.
- Carnahan JF, Anderson DJ, and Patterson PH (1991). Evidence that enteric neurons may derive from the sympathoadrenal lineage. *Dev Biol* 148:552-561.
- Chomczynski P and Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156-159.
- Claesson-Welsh L, Eriksson A, Westermarck B, and Heldin CH (1989). cDNA cloning and expression of the human A-type platelet-derived growth factor (PDGF) receptor establishes structural similarity to the B-type PDGF receptor. *Proc Natl Acad Sci USA* 86:4917-4921.
- DiCicco-Bloom E and Black IB (1988). Insulin growth factors regulate the mitotic cycle in cultured rat sympathetic neuroblasts. *Proc Natl Acad Sci USA* 85:4066-4070.
- Edsjö A, Hallberg B, Fagerström S, Larsson C, Axelson H, and Pålman S (2001). Differences in early and late responses between neurotrophin-stimulated *trkA*- and *trkC*-transfected SH-SY5Y neuroblastoma cells. *Cell Growth Differ* 12:39-50.

- Encinas M, Iglesias M, Liu Y, Wang H, Muhaisen A, Cena V, Gallego C, and Comella JX (2000). Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells. *J Neurochem* 75:991–1003.
- Encinas M, Iglesias M, Llecha N, and Comella JX (1999). Extracellular-regulated kinases and phosphatidylinositol 3-kinase are involved in brain-derived neurotrophic factor-mediated survival and neuritogenesis of the neuroblastoma cell line SH-SY5Y. *J Neurochem* 73:1409–1421.
- Francis NJ and Landis SC (1999). Cellular and molecular determinants of sympathetic neuron development. *Annu Rev Neurosci* 22:541–566.
- Fröstad B, Martinsson T, Tani E, Falkmer U, Darnfors C, Skoog L, and Kogner P (1999). The use of fine-needle aspiration cytology in the molecular characterization of neuroblastoma in children. *Cancer* 87:60–68.
- Gaetano C, Matsumoto K, and Thiele CJ (1991). Retinoic acid resistant neuroblastoma cells and the expression of insulin-like growth factor-II. *Prog Clin Biol Res* 366:165–172.
- George RE, Kenyon R, McGuckin AG, Kohl N, Kogner P, Christiansen H, Pearson AD, and Lunec J (1997). Analysis of candidate gene co-amplification with MYCN in neuroblastoma. *Eur J Cancer* 33:2037–2042.
- Gestblom C, Grynfeld A, Øra I, Örtoft E, Larsson C, Axelson H, Sandstedt B, Cserjesi P, Olson EN, and Pålman S (1999). The basic helix-loop-helix transcription factor dHAND, a marker gene for the developing human sympathetic nervous system, is expressed in both high- and low-stage neuroblastomas. *Lab Invest* 79:67–79.
- Gestblom C, Hoehner JC, Hedborg F, Sandstedt B, and Pålman S (1997). In vivo spontaneous neuronal to neuroendocrine lineage conversion in a subset of neuroblastomas. *Am J Pathol* 150:107–117.
- Godbout R and Squire J (1993). Amplification of a DEAD box protein gene in retinoblastoma cell lines. *Proc Natl Acad Sci USA* 90:7578–7582.
- Gordon-Weeks PR (1988). The ultrastructure of noradrenergic and cholinergic neurons in the autonomic nervous system. In: Björklund A, Hökfelt T, and Owman C, editors. *Handbook of chemical neuroanatomy*. Amsterdam: Elsevier, 117–181.
- Handler A, Lobo MD, Alonso FJ, Paino CL, and Mena MA (2000). Functional implications of the noradrenergic-cholinergic switch induced by retinoic acid in NB69 neuroblastoma cells. *J Neurosci Res* 60:311–320.
- Hedborg F, Lindgren PG, Johansson I, Kogner P, Samuelsson BO, Bekassy AN, Olsen L, Kreuger A, and Pålman S (1992). N-myc gene amplification in neuroblastoma: A clinical approach using ultrasound guided cutting needle biopsies collected at diagnosis. *Med Pediatr Oncol* 20:292–300.
- Hedborg F, Ohlsson R, Sandstedt B, Grimelius L, Hoehner JC, and Pålman S (1995). IGF2 expression is a marker for paraganglionic/SIF cell differentiation in neuroblastoma. *Am J Pathol* 146:833–847.
- Hill DP and Robertson KA (1997). Characterization of the cholinergic neuronal differentiation of the human neuroblastoma cell line LA-N-5 after treatment with retinoic acid. *Brain Res Dev Brain Res* 102:53–67.
- Hoehner JC, Gestblom C, Hedborg F, Sandstedt B, Olsen L, and Pålman S (1996a). A developmental model of neuroblastoma: Differentiating stroma-poor tumors' progress along an extra-adrenal chromaffin lineage. *Lab Invest* 75: 659–675.
- Hoehner JC, Olsen L, Sandstedt B, Kaplan DR, and Pålman S (1995). Association of neurotrophin receptor expression and differentiation in human neuroblastoma. *Am J Pathol* 147:102–113.
- Hoehner JC, Wester T, Pålman S, and Olsen L (1996b). Localization of neurotrophins and their high-affinity receptors during human enteric nervous system development. *Gastroenterology* 110:756–767.
- Judware R and Culp LA (1995). Over-expression of transfected N-myc oncogene in human SKNSH neuroblastoma cells down-regulates expression of beta 1 integrin subunit. *Oncogene* 11:2599–2607.
- Kaplan DR, Matsumoto K, Lucarelli E, and Thiele CJ (1993). Induction of TrkB by retinoic acid mediates biologic responsiveness to BDNF and differentiation of human neuroblastoma cells. *Eukaryotic Signal Transduction Group Neuron* 11:321–331.
- Kapur RP (1993). Contemporary approaches toward understanding the pathogenesis of Hirschsprung disease [published erratum appears in *Pediatr Pathol*. 1993;13:270]. *Pediatr Pathol* 13:83–100.
- Klein R, Conway D, Parada LF, and Barbacid M (1990). The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. *Cell* 61:647–656.
- Klein R, Parada LF, Coulier F, and Barbacid M (1989). trkB, A novel tyrosine protein kinase receptor expressed during mouse neural development. *Embo J* 8:3701–3709.
- Kohl NE, Kanda N, Schreck RR, Bruns G, Latt SA, Gilbert F, and Alt FW (1983). Transposition and amplification of oncogene-related sequences in human neuroblastomas. *Cell* 35:359–367.
- Lasorella A, Nosedà M, Beyna M, and Iavarone A (2000). Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins. *Nature* 407:592–598.
- Lavenius E, Gestblom C, Johansson I, Nånberg E, and Pålman S (1995). Transfection of TRK-A into human neuroblastoma cells restores their ability to differentiate in response to nerve growth factor. *Cell Growth Differ* 6:727–736.
- Lesser SS, Sherwood NT, and Lo DC (1997). Neurotrophins differentially regulate voltage-gated ion channels. *Mol Cell Neurosci* 10:173–183.
- Martin-Zanca D, Oskam R, Mitra G, Copeland T, and Barbacid M (1989). Molecular and biochemical characterization of the human trk proto-oncogene. *Mol Cell Biol* 9:24–33.
- Martinsson T, Sjöberg RM, Hedborg F, and Kogner P (1995). Deletion of chromosome 1p loci and microsatellite instability in neuroblastomas analyzed with short-tandem repeat polymorphisms. *Cancer Res* 55:5681–5686.
- Matsumoto K, Wada RK, Yamashiro JM, Kaplan DR, and Thiele CJ (1995). Expression of brain-derived neurotrophic factor and p145TrkB affects survival, differentiation, and invasiveness of human neuroblastoma cells. *Cancer Res* 55:1798–1806.

- Matsushima H and Bogenmann E (1993). Expression of *trkA* cDNA in neuroblastomas mediates differentiation in vitro and in vivo. *Mol Cell Biol* 13:7447–7456.
- Matthay KK, Villablanca JG, Seeger RC, Stram DO, Harris RE, Ramsay NK, Swift P, Shimada H, Black CT, Brodeur GM, Gerbing RB, and Reynolds CP (1999). Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-*cis*-retinoic acid. Children's Cancer Group. *N Engl J Med* 341:1165–1173.
- Middlemas DS, Lindberg RA, and Hunter T (1991). *trkB*, A neural receptor protein-tyrosine kinase: Evidence for a full-length and two truncated receptors. *Mol Cell Biol* 11:143–153.
- Mintz CD, Andrews PC, and Dixon JE (1986). Characterization, sequence, and expression of the cloned human neuropeptide Y gene. *J Biol Chem* 261:11974–11979.
- Nakagawara A, Azar CG, Scavarda NJ, and Brodeur GM (1994). Expression and function of *TRK-B* and *BDNF* in human neuroblastomas. *Mol Cell Biol* 14:759–767.
- Ørtoft E, Pålman S, Andersson G, Parrow V, Betsholtz C, and Hammerling U (1993). Human *GAP-43* expression: Multiple start sites for initiation of transcription in differentiating human neuroblastoma cells. *Mol Cell Neurosci* 4:549–561.
- Pålman S and Hedborg F (2000). Development of the neural crest and sympathetic nervous system. In: Pålman S and Hedborg F, editors. Amsterdam: Elsevier Science B. V., 9–19.
- Pålman S, Johansson I, Westermark B, and Nister M (1992). Platelet-derived growth factor potentiates phorbol ester-induced neuronal differentiation of human neuroblastoma cells. *Cell Growth Differ* 3:783–790.
- Pålman S, Meyerson G, Lindgren E, Schalling M, and Johansson I (1991). Insulin-like growth factor I shifts from promoting cell division to potentiating maturation during neuronal differentiation. *Proc Natl Acad Sci USA* 88:9994–9998.
- Pålman S, Odelstad L, Larsson E, Grotte G, and Nilsson K (1981). Phenotypic changes of human neuroblastoma cells in culture induced by 12-*O*-tetradecanoyl-phorbol-13-acetate. *Int J Cancer* 28:583–589.
- Pålman S, Ruusala AI, Abrahamsson L, Mattsson ME, and Esscher T (1984). Retinoic acid-induced differentiation of cultured human neuroblastoma cells: A comparison with phorbol ester-induced differentiation. *Cell Differ* 14:135–144.
- Patterson PH (1990). Control of cell fate in a vertebrate neurogenic lineage. *Cell* 62:1035–1038.
- Schwab M (2000). *MYCN* amplification in neuroblastoma. In: Schwab M, editor. Amsterdam: Elsevier Science B. V., 75–83.
- Shohet JM, Hicks MJ, Plon SE, Burlingame SM, Stuart S, Chen SY, Brenner MK, and Nuchtern JG (2002). Minichromosome maintenance protein *MCM7* is a direct target of the *MYCN* transcription factor in neuroblastoma. *Cancer Res* 62:1123–1128.
- Stemple DL, Mahanthappa NK, and Anderson DJ (1988). Basic FGF induces neuronal differentiation, cell division, and NGF dependence in chromaffin cells: A sequence of events in sympathetic development. *Neuron* 1:517–525.
- Tso JY, Sun XH, Kao TH, Reece KS, and Wu R (1985). Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: Genomic complexity and molecular evolution of the gene. *Nucleic Acids Res* 13:2485–2502.
- van der Hoorn FA, Boomsma F, Man in 't Veld AJ, and Schalekamp MA (1989). Determination of catecholamines in human plasma by high-performance liquid chromatography: Comparison between a new method with fluorescence detection and an established method with electrochemical detection. *J Chromatogr* 487:17–28.
- Verdi JM and Anderson DJ (1994). Neurotrophins regulate sequential changes in neurotrophin receptor expression by sympathetic neuroblasts. *Neuron* 13:1359–1372.
- Yokomori K, Tsuchida Y, and Saito S (1983). Tyrosine hydroxylase and choline acetyltransferase activity in human neuroblastoma: Correlations with clinical features. *Cancer* 52:263–272.
- Zackenfels K, Oppenheim RW, and Rohrer H (1995). Evidence for an important role of *IGF-I* and *IGF-II* for the early development of chick sympathetic neurons. *Neuron* 14:731–741.