RESULTADOS

6.1 Las ERK MAPKs y la PI 3-K regulan la neuritogénesis y superviencia de la línea de neuroblastoma humano SH-SY5Y

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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

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Abstract: Retinoic acid (RA) induces the differentiation of many cell lines, including those derived from neuroblastoma. RA treatment of SH-SY5Y cells induces the appearance of functional Trk B and Trk C receptors. Acute stimulation of RA-predifferentiated SH-SY5Y cells with brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), or neurotrophin 4/5 (NT-4/5), but not nerve growth factor (NGF), induces Trk autophosphorylation, followed by phosphorylation of Akt and the extracellular signal-regulated kinases (ERKs) 1 and 2. In addition, BDNF, NT-3, or NT-4/5, but not NGF, promotes cell survival and neurite outgrowth in serum-free medium. The mitogen-activated protein kinase and ERK kinase (MEK) inhibitor PD98059 blocks BDNF-induced neurite outgrowth and growth-associated protein-43 expression but has no effects on cell survival. On the other hand, the phosphatidylinositol 3-kinase inhibitor LY249002 reverses the survival response elicited by BDNF, leading to a cell death with morphological features of apoptosis. **Key Words:** Trk—Neurotrophin—Neuroblastoma—Neurodegeneration.
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Neurotrophins constitute a family of structurally related growth factors that regulate neuronal survival, maturation, and function of several populations of neurons. This family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and neurotrophin-6 (Lewin and Barde, 1996). Increasing evidence suggests that the survival of defined populations of neurons is promoted by specific neurotrophins (Davies, 1994, 1996). The biological effects of neurotrophins on neuronal cells are mediated by two classes of specific receptors. The first, $p75^{NTR}$, does not show any binding preference for the different neurotrophins (reviewed by Bothwell, 1995) and has been recently found to be involved in the regulation of neuronal survival due to its ability to induce apoptosis (Bunone et al., 1997; reviewed by Kaplan and Miller, 1997). The second, the tropomyosin receptor kinase (Trk) family of tyrosine kinase receptors, binds neurotrophins in a specific manner. Trk A is predominantly activated by NGF, Trk B by BDNF or NT-4/5, and Trk C by NT-3. NT-3, however, can also activate Trk B and Trk A to a lesser extent than their specific ligands (Barbacid, 1995; Chao and Hempstead, 1995).

Continuously dividing cell lines such as the neuroblastoma SH-SY5Y have been widely used as a model to study neurodegenerative disorders such as Alzheimer's (Lambert et al., 1994; Li et al., 1996; Hartmann et al., 1997; Peraus et al., 1997) and Parkinson's (Sheehan et al., 1997) diseases. Neuroblastoma cell lines differentiate into cells that are biochemically, ultrastructurally, and electrophysiologically similar to neurons (Abemayor and Sidell, 1989) after being treated with different agents such as neurotrophic factors (Kaplan et al., 1993), retinoic acid (RA) or phorbol ester (Pahlman et al., 1995), or staurosporine (Jalava et al., 1992, 1993). RA causes the differentiation of SH-SY5Y cells and mediates biological responsiveness of the cells to the Trk B ligands BDNF and NT-3 by stimulating the expression of Trk B

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Abbreviations used: BDNF, brain-derived neurotrophic factor; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; GAP-43, growth-associated protein-43; MAPK, mitogen-activated protein kinase; MEK, MAPK and ERK kinase; NGF, nerve growth factor; NT-3, neurotrophin-3; NT-4/5, neurotrophin-4/5; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI 3-K, phosphatidylinositol 3-kinase; PLCγ, phospholipase Cγ; RA, retinoic acid; SDS, sodium dodecyl sulfate; Trk, tropomyosin receptor kinase.

receptors (Kaplan et al., 1993). Several signal transduction pathways are used by the neurotrophins to mediate cellular events such as survival and differentiation (reviewed by Segal and Greenberg, 1996; Kaplan and Miller, 1997). Some proteins are known to associate with NGF-activated Trk; among them are Shc, phospholipase Cγ1 (PLCγ1), phosphatidylinositol 3-kinase (PI 3-K), and SHP. These proteins are thought to couple Trk to different intracellular signaling pathways (Barbacid, 1995). Thus, for example, tyrosine phosphorylation of Shc due to its interaction to Trk results in a rapid activation of the Ras/mitogen-activated protein kinase (MAPK) pathway (reviewed by Segal and Greenberg, 1996). The use of neutralizing Ras antibodies (Nobes and Tolkovsky, 1995), dominant-positive and -negative variants of MAPK and extracellular signal-regulated kinase (ERK) kinase (MEK) (Cowley et al., 1994), or the selective MEK inhibitor PD98059 (Pang et al., 1995) has shown that Ras and MEK are necessary for NGF-induced neuritogenesis or survival in PC12 cells and rat sympathetic neurons (reviewed by Kaplan and Stephens, 1994; Kaplan and Miller, 1997). However, PD98059 was unable to block NGF-mediated neurite outgrowth from chick sensory and sympathetic neurons, indicating that MEK and MAPK might not be necessary for neuritogenesis in peripheral neurons (Klinz et al., 1996). NGF-activated Trk can also stimulate the PI 3-K/Akt pathway (Ohmichi et al., 1992; Burgering and Coffer, 1995; Franke et al., 1995). This pathway has been involved in neurotrophic factor-induced cell survival in different systems (Yao and Cooper, 1995, 1996; Vemuri and McMorris, 1996; D'Mello et al., 1997; Dudek et al., 1997; Miller et al., 1997).

The results described above have been mainly obtained using the PC12 cell line, which responds to NGF through Trk A (Greene and Tischler, 1976). Little information is available about the intracellular pathways triggered by Trk B or Trk C as a result of activation by BDNF, NT-3, or NT-4/5. Furthermore, few results have been reported about the role of the PI 3-K/Akt or Ras/MAPK pathway on the survival and differentiation of BDNF-dependent neurons. In the present report we tried to elucidate the role of these pathways in neuritogenesis and cell survival of BDNF-dependent SH-SY5Y cells. We have observed that BDNF is able to activate both pathways (PI 3-K/Akt and Ras/MAPK) in SH-SY5Y cells that have been preexposed to RA for 5 days. Moreover, we report that PI 3-K/Akt is involved in BDNF-mediated cell survival, whereas ERK/MAPK is involved in BDNF-induced neuritogenesis.

MATERIALS AND METHODS

Materials

all-*trans*-RA, L-α-phosphatidylinositol, and L-α-phosphatidylserine were purchased from Sigma (St. Louis, MO, U.S.A.). Human recombinant BDNF, NT-3, and NT-4/5 were from Alomone Laboratories (Jerusalem, Israel). NGF 7S was purified at our laboratory from mouse submaxillary glands as previously described (Mobley et al., 1976). [γ-^{32}P]ATP was from Amersham Pharmacia Biotech (Amersham Ibérica, Madrid, Spain). The MEK inhibitor PD98059 and the PI 3-K inhibitor LY294002 were from Calbiochem-Novabiochem (Läufelfingen, Switzerland). Both drugs were dissolved in dimethyl sulfoxide; the final concentration of this solvent never exceeded 0.1% (vol/vol).

The 4G10 anti-phosphotyrosine and the 203 anti-pan-Trk antibodies were generous gifts of Dr. D. Martin-Zanca (Salamanca, Spain). Anti-phospho-ERK and anti-phospho-Akt antibodies were from New England Biolabs (Beverly, MA, U.S.A.). Anti-Akt, anti-Trk A, anti-Trk B, and anti-Trk C were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-pan-ERK antibody was from Transduction Laboratories (Lexington, KY, U.S.A.). Rabbit anti-mouse IgG was from Sigma, and peroxidase-conjugated secondary antibodies were from Sigma or Amersham Ibérica.

Cell culture

The SH-SY5Y neuroblastoma cell line was from ATCC. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 m*M* L-glutamine, penicillin (20 units/ml), and streptomycin (20 mg/ml) (basal DMEM) and 15% (vol/vol) heat-inactivated fetal calf serum (GIBCO, Gaithersburg, MD, U.S.A.). Cells were maintained at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO_2. all-*trans*-RA was added to the medium to a final concentration of 10 μ*M*. The medium was changed every 3 days. For cell survival, neurite outgrowth, and Hoechst staining, cells were plated in 35-mm-diameter (Corning, Corning, NY, U.S.A.) at an initial density of 5×10^4–1×10^5 cells per well.

Survival assays

The number of living cells was established after scraping the cells from the culture plate, staining them with trypan blue (Sigma), and counting them in the hemocytometer (Bürker). Cells were treated for 5 days with RA at 10 μ*M*, washed three times with basal DMEM, and switched to basal DMEM containing 2 n*M* neurotrophin plus the indicated drugs for different additional intervals. Each experiment was performed in quadruplicate. Before switching to neurotrophin-containing medium, four different wells (each from a different six-well culture plate) were counted, and the resulting value was defined as 100% survival, i.e., the initial number of cells. Results were therefore expressed as a mean ± SEM percentage of this value. Differences were considered to be significant for $p < 0.05$. The morphological features of the cell death caused by LY249002 were examined by Hoechst 33258 staining. After 48 h in the presence of BDNF alone or BDNF plus LY249002 (1, 5, and 10 μ*M*), cells were fixed with paraformaldehyde for 15 min and then stained with Hoechst 33258 [0.05 μg/ml in phosphate-buffered saline (PBS)] for 30 min. They were rinsed twice with PBS, mounted with Fluoprep (BioMerieux, Marcy l'Etoile, France), and counted using an Olympus fluorescence microscope equipped with UV illumination. Apoptotic cell death was expressed as the mean ± SEM value of apoptotic cells, and differences were considered to be significant when $p < 0.05$.

Evaluation of neuritogenesis

SH-SY5Y cells were treated for 5 days with 10 μ*M* RA. Then they were washed three times with basal DMEM and switched to basal DMEM containing one of the neurotrophins at a 2 n*M* concentration and, when required, the indicated drugs. After 24 h, cultures were observed in a phase-contrast microscope (Olympus DXC-107AP) connected through a video

camera to a Pentium MMX PC running an adequate image analyzer software (PC-Image; Foster Findlay Associates Ltd., Newcastle upon Tyne, U.K.). The cell bodies and neurites present in 10 randomly selected fields were counted. The ratio between cell bodies and neurites was calculated yielding the average of neurites per neuron and was expressed as the mean ± SEM value. Differences were considered to be significant when $p < 0.05$.

Trk immunoprecipitation experiments

At the end of each RA treatment period, cells were washed three times and incubated for an additional 3 h in basal DMEM. Cells were then acutely stimulated with the corresponding neurotrophin (2 n*M*) for 5 min, rinsed rapidly with ice-cold PBS, and solubilized with immunoprecipitation buffer [20 m*M* Tris (pH 7.4), 150 m*M* NaCl, 2 m*M* EDTA, 1 m*M* EGTA, 1% Nonidet P-40, 1 m*M* sodium orthovanadate, 1 m*M* phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2 m*M* benzamidine, and 20 μg/ml leupeptin] for 15 min. Cells were then scraped off and orbitally rotated for an additional 15-min period at 4°C. Nuclei and cellular debris were removed by microfuge centrifugation at 10,000 *g* and 4°C for 15 min. Protein concentration in the resulting supernatant was quantified by a modified Lowry assay as described by the manufacturer (DC protein assay; Bio-Rad, Hercules, CA, U.S.A.).

Immunoprecipitation assays were performed in nondenaturing conditions. Five hundred micrograms of total protein was incubated with an anti-pan-Trk polyclonal antibody (203) at 4°C either for 1 h or overnight. The immunocomplexes were collected with protein A-Sepharose, washed two times with immunoprecipitation buffer and once with PBS containing 1 m*M* sodium orthovanadate, and finally boiled for 5 min. Samples were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene difluoride Immobilon-P transfer membrane filters (Millipore, Bedford, MA, U.S.A.), and probed with anti-phosphotyrosine antibody as described below.

For immunoprecipitates using the anti-Trk A, anti-Trk B, or anti-Trk C antibodies, the procedure was similar to the one described above except for the immunoprecipitation buffer [1% Nonidet P-40, 20 m*M* Tris (pH 7.4), 10 m*M* EGTA, 40 m*M* β-glycerophosphate, 2.5 m*M* $MgCl_2$, 2 m*M* orthovanadate, 1 m*M* dithiothreitol, 1 m*M* phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin], the amount of total protein used (∼1 mg per condition), and the amount of antibody (8 μg per condition).

Western blot

For MAPK and Akt immunodetection, cells were treated with 10 μ*M* RA for 5 days and then serum-starved for 5 h. When needed, a 30-min preincubation step with PD98059, LY249002, or serum-free medium without drugs was included. Cells were then stimulated for 5 min with the corresponding neurotrophin at 2 n*M,* rinsed rapidly in ice-cold PBS, and lysed in 2% SDS and 125 m*M* Tris (pH 6.8) buffer. Lysates were sonicated, and protein was quantified by means of the Bio-Rad DC protein assay. Immunoprecipitates or cell lysates (50 μg of protein per lane) were resolved in SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride membrane filters using a Pharmacia semidry Trans-Blot according to the manufacturer's instructions. Membranes were blocked with Tris-buffered saline with Tween 20 [20 m*M* Tris-HCl (pH 7.4), 150 m*M* NaCl, and 0.05% Tween 20] containing 5% nonfat dry milk (5% bovine serum albumin when anti-phosphotyrosine antibody was used) for 1 h at room temperature. Membranes were probed with the different antibodies at the dilutions recommended by the manufacturer for 1 h at room temperature and subsequently incubated with peroxidase-conjugated secondary antibodies. Blots were finally developed with the enhanced chemiluminescence western blotting detection system (Amersham, Little Chalfont, U.K.). Alternatively, the SuperSignal chemiluminescent substrate (Pierce, Rockford, IL, U.S.A.) was used.

When needed, membranes were stripped with 100 m*M* 2-mercaptoethanol, 2% SDS, and 62.5 m*M* Tris-HCl (pH 6.8) for 30 min at 70°C. Filters were reprobed using anti-pan-ERK, anti-pan-Akt, or anti-pan-Trk antibodies.

PI 3-K activity assay

Cells were incubated for 5 days in the presence of 10 μ*M* RA, washed, and subsequently incubated with basal DMEM for 5 h. Cells were then stimulated for 1 min with 2 n*M* BDNF, rinsed rapidly in ice-cold PBS, and solubilized in 1% Nonidet P-40 buffer [20 m*M* Tris (pH 7.4), 150 m*M* NaCl, 5 m*M* EDTA, 1% Nonidet P-40, 1 m*M* sodium orthovanadate, 1 m*M* phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2 m*M* benzamidine, and 20 μg/ml leupeptin] for 15 min. Cells were then scraped off from the dishes, orbitally rotated for 15 min, and centrifuged at 10,000 *g* for an additional 15 min to remove cellular debris. Protein in the supernatant was quantified, and 500 μg of protein was immunoprecipitated overnight at 4°C with anti-phosphotyrosine antibody (4G10). Immunocomplexes were collected with protein A-Sepharose preconjugated with an anti-mouse IgG (Sigma) and sequentially washed with lysis buffer, LiCl buffer [100 m*M* Tris (pH 7.5), 0.5 *M* LiCl, 1 m*M* EDTA, and 1 m*M* sodium orthovanadate], and TNE buffer [25 m*M* Tris (pH 7.5), 100 m*M* NaCl, and 1 m*M* EDTA]. Immunocomplexes were incubated with a mixture of L-α-phosphatidylinositol and L-α-phosphatidylserine (final concentration, 0.5 mg/ml each) and 10 μCi of [γ-^{32}P]ATP. LY249002 or TNE buffer alone was added at the indicated concentrations directly to the reaction mixture. Incubation was allowed to proceed for 20 min at room temperature. Phosphorylated lipids were then extracted and resolved by TLC using *n*-propanol/water/acetic acid (66:33:2, by volume) as a solvent. Radioactive spots were detected by autoradiography by exposing the TLC plate to Fuji Medical x-ray film (Fuji Photo Film Co. Ltd., Tokyo, Japan) overnight at −70°C.

RT-PCR

A semiquantitative RT-PCR assay was used to analyze the expression pattern of growth-associated protein-43 (GAP-43). One microgram of total RNA (Chomczynski and Sacchi, 1987) was treated with 2 U of RNase-free DNase I (Pharmacia) and reverse-transcribed using 1 nmol of random hexamers (Boehringer Mannheim) and 200 U of Moloney murine leukemia virus reverse transcriptase (Promega) for 1 h at 37°C. Ten nanograms of cDNA was used to perform a multiplex PCR amplification, with each of the GAP-43 set of primers at 400 n*M* and each of the housekeeping L27 ribosomal proteins at 40 n*M* as an internal control. Samples were subjected to 32 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 45 s on a Perkin Elmer thermal cycler, with hot start at 94°C. Products were analyzed on 2% ethidium bromide-stained gels. Care was taken to arrest the amplification to the linear phase. To achieve this, the amount of product was plotted against number of cycles and amount of starting sample. Primer sequences were as follows: GAP-43 forward (GAP-43-F), AGGCCGCAACCAAAATTCAGG; GAP-43 reverse (GAP-43-R), TCCGTTGGAGGCTGGGCTGTT; L27 forward (L27F), AGCTGTCATCGTGAAGAACAT; L27 reverse (L27R), CTTGGCGATCTTCTTCTTGCC.

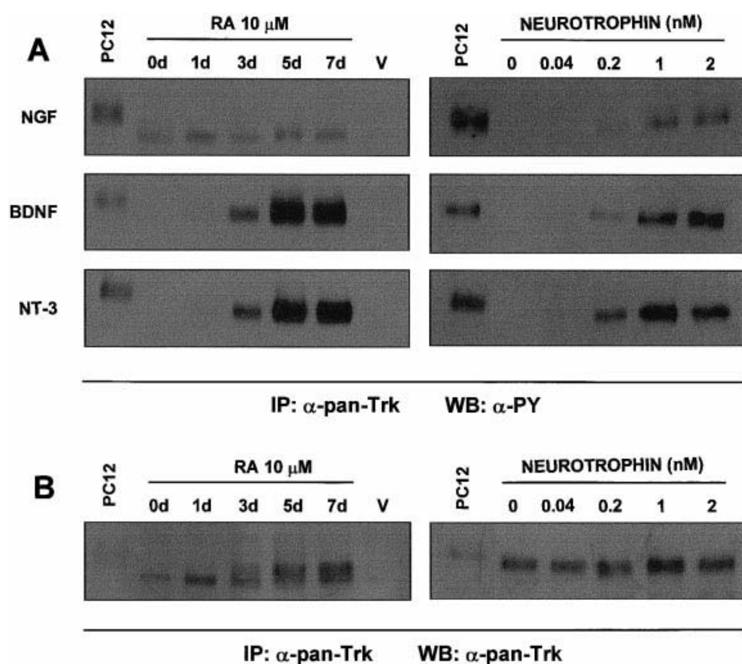


FIG. 1. A, left panel: RA induces the appearance of functional Trk B and Trk C but not Trk A receptors. Cells were cultured in the presence of 10 *μM* RA for the indicated times and then stimulated (5 min, 2 n*M*) with NGF (**top**), BDNF (**middle**), or NT-3 (**bottom**). Trk receptors were immunoprecipitated (IP) with an anti-pan-Trk antiserum, and tyrosine phosphorylation was analyzed by western blotting (WB). V, cells treated for 7 days with vehicle, 0.2% dimethyl sulfoxide. **A, right panel:** Dose–response curve of neurotrophin-induced Trk phosphorylation. Cells were incubated for 5 days with 10 *μM* RA and then stimulated with the indicated concentrations of each neurotrophin for 5 min. Trk receptors were immunoprecipitated with an anti-pan-Trk antiserum, and tyrosine phosphorylation was analyzed by western blotting. PC12 cells stimulated with 2 n*M* NGF were used as a positive control. **B:** Filters corresponding to the NT-3 condition in A were stripped and reprobed with the anti-pan-Trk antiserum as described in Materials and Methods to assess the total quantity of Trk. Note that in the left panel there is an increase in the quantity of Trk over time of RA treatment.

RESULTS

RA induces the appearance of functional Trk B and Trk C but not Trk A in SH-SY5Y cells

As previously reported (Pahlman et al., 1984), we observed that SH-SY5Y cells reduced their growth rate and differentiated toward a neuronal phenotype when exposed to 10 *μM* RA (data not shown). Kaplan et al. (1993) reported that RA induces Trk B expression on SH-SY5Y cells, making them responsive to BDNF. Based on this information we decided to study the appearance of functionally active Trk receptors on the cell surface during RA treatment. We first incubated the cells for different intervals in the presence of 10 *μM* RA, and then we acutely stimulated them with NGF, BDNF, NT-3, or NT-4/5. PC12 cells stimulated with NGF were included as a positive control. Cells were lysed, and the extracts were immunoprecipitated with an anti-pan-Trk antiserum (G203). Immunoprecipitates were subsequently subjected to western blot using an anti-phosphotyrosine-specific antibody (4G10). In RA-untreated cells, NGF-mediated Trk autophosphorylation was barely detectable. After 1 day of RA treatment, a slight increase of Trk A phosphorylation was observed, which remained nearly constant up to day 7 (Fig. 1A, left panel). In contrast, naive cells did not show significant Trk phosphorylation when treated with BDNF. However, the induction of Trk phosphorylation by BDNF greatly increased on RA treatment, being evident after 3 days of treatment, reaching a peak after 5 days, and remaining constant up to day 7 (Fig. 1A, left panel). The kinetics and intensity of NT-3-induced Trk activation were very similar to those observed for BDNF (Fig. 1A, left panel). Reprobing those filters with a pan-Trk antibody (203) showed that the increase in the phosphorylation signal correlated with an increase of the total amount of Trk. In addition, bands with reduced mobility appeared during the RA treatment, possibly due to progressive glycosylation of the protein (Fig. 1B, left panel). Of note is that the apparent molecular weight of the Trk receptor from PC12 cells was slightly higher than the one observed for human Trks. This phenomenon has been previously described and is due to interspecific differences in glycosylation (Kaplan et al., 1991; Nakagawara et al., 1994).

Another important aspect was to elucidate whether NT-3 was acting through its cognate receptor Trk C or through Trk B, because this neurotrophin can bind Trk B with low affinity in a neuronal context (Ip et al., 1993). For this purpose, we tested the minimal concentration of NT-3 required to induce Trk autophosphorylation. Concentrations of 0.2 n*M* NT-3 were enough to stimulate Trk phosphorylation, thus reflecting a highly specific interaction between NT-3 and the Trk receptor (Fig. 1A, right panel). This threshold concentration was similar to the one observed for NGF, BDNF (Fig. 1A, right panel), and NT-4/5 (data not shown). Moreover, immunoprecipitates using either anti-Trk B- or anti-Trk C-specific antibodies revealed that NT-3 induced autophosphorylation of Trk C but not of Trk B (Fig. 2). Taken together, these data strongly suggest that RA is promoting the appearance of functional Trk B and Trk C receptors.

BDNF, NT-4/5, and NT-3 but not NGF support differentiation and survival of RA-treated SH-SY5Y cells

To test whether these Trk receptors were able to mediate any biological effect, we cultured SH-SY5Y cells for 5 days in the presence of 10 *μM* RA, and then we switched them to serum-free medium supplemented with each of the neurotrophins at 2 n*M*. After an additional

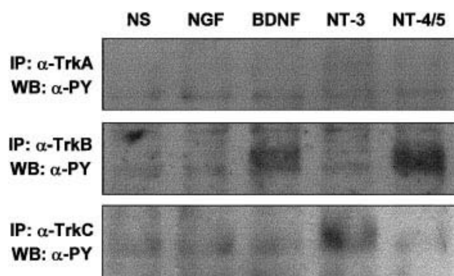


FIG. 2. Neurotrophin-induced Trk phosphorylation is mediated by specific Trk receptors. Cells were treated with 10 μM RA for 5 days and then were stimulated with the indicated neurotrophins (5 min, 2 nM). Trk receptors were immunoprecipitated (IP) with specific antibodies against Trk A (**top**), Trk B (**middle**), or Trk C (**bottom**), and tyrosine phosphorylation was analyzed by western blot (WB). Note that NGF-induced Trk A phosphorylation is not detectable, probably owing to a reduced immunoprecipitation efficiency of this antibody with respect to the 203 antiserum. NS, nonstimulated cells.

48 h in culture, we evaluated cell survival by means of the trypan blue staining. We defined 100% survival as the amount of living cells present at the time of switching from RA to the neurotrophin, that is, after 5 days in the presence of RA (referred to as initial cells). We observed a potent cell survival effect when either BDNF or NT-4/5 was present in the medium. NT-3 also supported cell survival but to a significantly lesser extent, whereas NGF had no significant effect (Fig. 3A). These results were consistent with the expression of Trk receptors after RA treatment reported above (Fig. 1).

An additional effect of BDNF and NT-4/5 was an enhancement of neuritogenesis. After 24 h in the presence of the neurotrophin, RA-pretreated cells acquired rounded, phase-bright bodies and displayed long neurites. Cells were scattered over the culture plate, and their neurites tended to connect to each other, forming a neuritic network on the surface of the culture plate (Fig. 3B and C). NT-3 induced a modest, although significant, degree of differentiation. However, the percentage of cells exhibiting neuritic processes was lower than that observed with BDNF or NT-4/5 (Fig. 3B and C). Finally, NGF or cultures without neurotrophin appeared to undergo little if any neuritic outgrowth (Fig. 3B and C).

BDNF, NT-3, and NT-4/5 but not NGF activate the MAPK and PI 3-K/Akt pathways

To elucidate which intracellular pathways could be involved in the biological effects of BDNF, NT-3, and NT-4/5, we assessed the degree of activation of the Ras/MAPK and PI 3-K/Akt pathways after neurotrophin stimulation. These pathways are activated on neurotrophin activation of Trk receptors and have been shown to be relevant for neuronal survival and differentiation (Kaplan and Miller, 1997). SH-SY5Y cells treated for 5 days with RA were acutely stimulated (5 min) with 2 nM NGF, BDNF, NT-3, or NT-4/5. The phosphorylation of ERK1 and 2 was assessed by means of western blot using an anti-phospho-ERK-specific antibody because a good correlation between the activity of this enzyme and its state of tyrosine phosphorylation has been reported (Egea et al., 1998, 1999). BDNF, NT-3, and NT-4/5 promoted tyrosine phosphorylation of ERK 1 and 2, whereas NGF did not (Fig. 4). Reprobing of the membrane with an anti-pan-ERK antibody showed that protein loading between the different lanes was similar. In our system, this antibody failed to recognize ERK1, but the total amount of ERK2 was similar for all the gel lanes (Fig. 4).

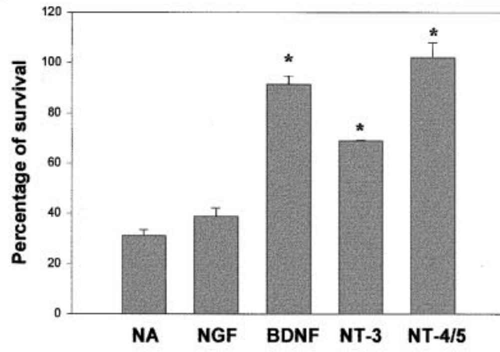
Akt phosphorylation was assessed in the same cellular extracts using a specific anti-phospho-Akt-specific antibody. The results were similar to the ones obtained for ERKs. That is, BDNF, NT-3, or NT-4/5, but not NGF, induced a strong phosphorylation of Akt (Fig. 4). Reprobing of the membrane with an anti-pan-ERK antibody showed that protein loading between the different lanes was similar (Fig. 4). Therefore, the activation of both ERK 1 and 2 and Akt in response to neurotrophins showed a good correlation with Trk autophosphorylation.

The MEK inhibitor PD98059 blocks BDNF-mediated neuritogenesis

As has been described in other systems, we have observed that BDNF activates the MAPK pathway. This pathway has been shown to be involved in NGF-induced neuritogenesis in PC12 pheochromocytoma cells (Cowley et al., 1994) and also in neurite outgrowth induced by insulin-like growth factor-I on SH-SY5Y cells (Kim et al., 1997). Based on these previous results, we studied the neuritogenic effect of BDNF in correlation with ERK activation in our system by using the MEK-specific inhibitor PD98059. MEK is a dual-specificity protein kinase that can activate ERKs by phosphorylating them on both threonine and tyrosine residues. PD98059 blocks the activation of MEK without affecting other known serine/threonine kinases or tyrosine kinases (Dudley et al., 1995; Pang et al., 1995). RA-predifferentiated SH-SY5Y cells were exposed to increasing concentrations (2, 20, and 50 μM) of PD98059 in the presence of BDNF. After 24 h of treatment, neurite outgrowth was scored. Exposure to PD98059 caused cells to become flattened, exhibit short processes, and aggregate (Fig. 5B), whereas in cultures without PD98059 cells appeared to extend long neurites and scatter over the culture plate surface (Fig. 5A). At the concentrations assayed, PD98059 caused a significant dose-dependent reduction of the average number of neurites per cell (Fig. 5C). This reduction correlated with a decreased tyrosine phosphorylation of the ERKs and MAPK after exposure to PD98059, as revealed by western blotting (Fig. 5D). Stripping and reprobing with an anti-pan-ERK antibody showed no differences in the total amount of ERK 2, indicating that such a decrease was not due to gel loading differences.

The effects of PD98059 on neuronal survival were evaluated by culturing RA-predifferentiated SH-SY5Y cells with BDNF plus PD98059 in serum-free medium

A



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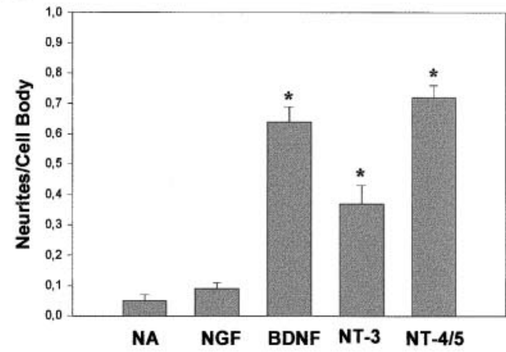

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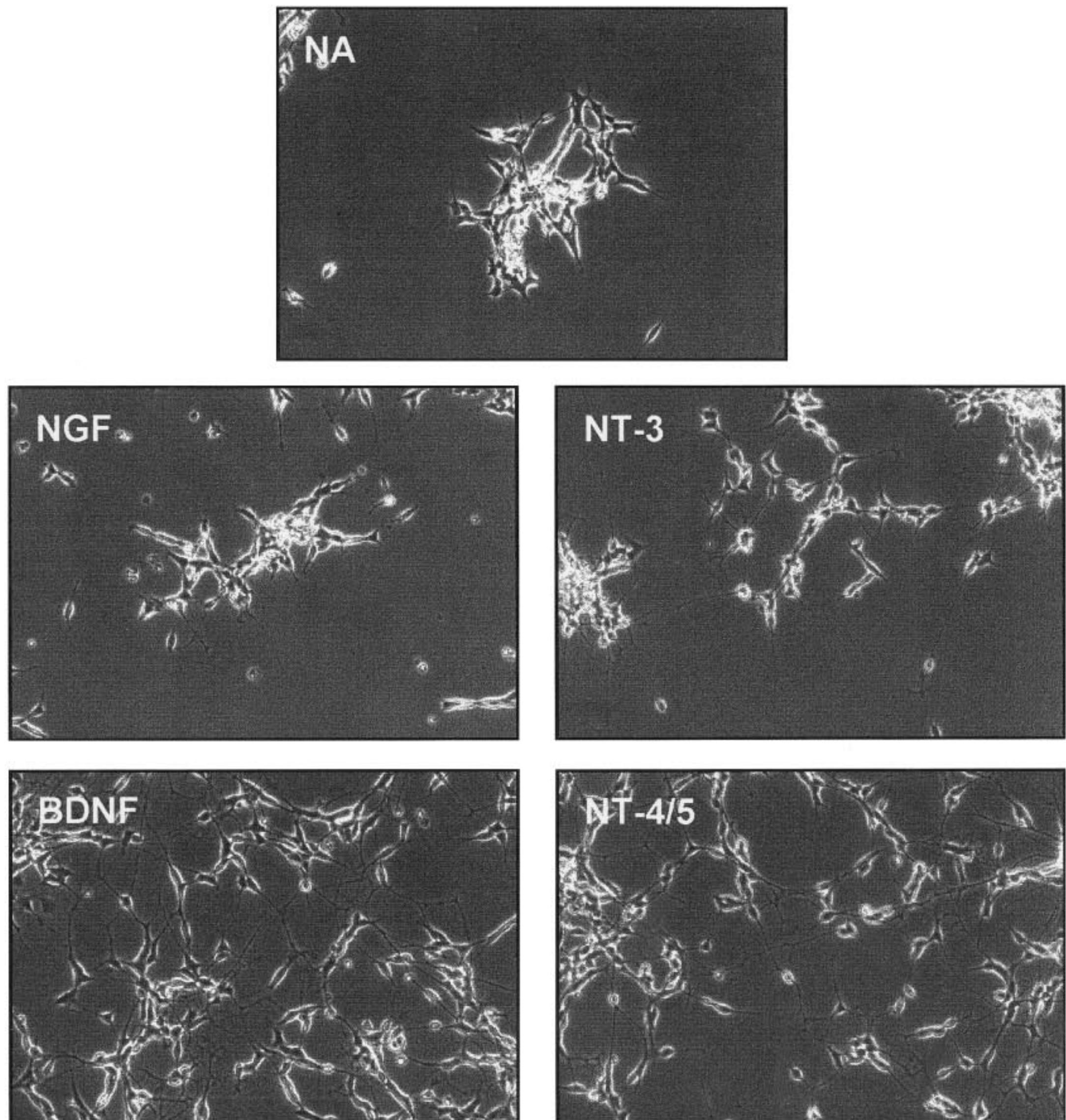


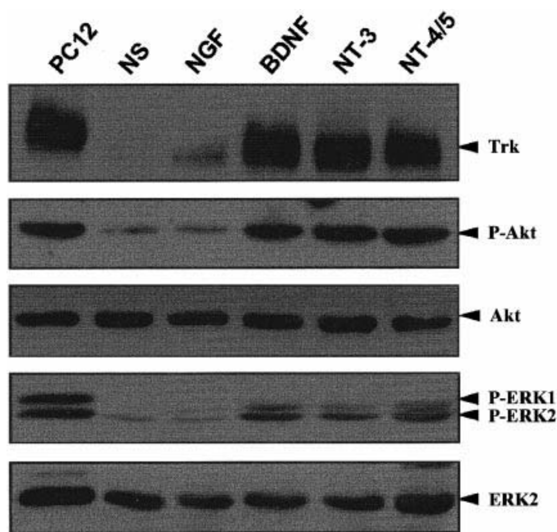


FIG. 4. Western blot of relevant phosphoproteins activated after neurotrophin stimulation. Cells were cultured for 5 days with 10 μM RA and then exposed for 5 min to the indicated neurotrophin (2 n*M*). Trk receptors were immunoprecipitated and probed with an anti-phosphotyrosine antibody as described in Materials and Methods. The phosphorylated forms of Akt (P-Akt) and ERKs 1 (P-ERK1) and 2 (P-ERK2) were detected with specific antibodies. Reprobing with an anti-pan-ERK antibody (ERK2), which fails to recognize ERK1 in our system, and anti-pan-Akt (Akt) was used as controls of protein loading. NS, nonstimulated cells. A lane containing lysates from PC12 cells stimulated for 5 min with 2 n*M* NGF was included as a positive control.

for 48 h. No significant differences between control and PD98059-treated cells were found (data not shown). Accordingly, PD98059 did not show any significant effect on Akt phosphorylation (data not shown).

BDNF induces GAP-43 expression through a MEK-dependent mechanism

GAP-43 is the most abundant neuron-specific protein in the growth cones, and its expression is regulated during neuronal differentiation (Lavenius et al., 1994; Kim et al., 1997). Because BDNF showed neurite-promoting effects in RA-treated SH-SY5Y cells, we examined the expression of GAP-43 by semiquantitative RT-PCR. Pretreatment with 10 μM RA had no effect on GAP-43 expression. However, after 6 h in the presence of BDNF, GAP-43 expression was dramatically increased. This increase was completely inhibited by PD98059, suggesting that the Ras/MAPK pathway is necessary for the expression of this neuronal marker (Fig. 6). The expression was attenuated after 12 h, but the blocking effect of PD98059 was persistent. Finally, parallel cultures maintained with RA for the total length of the experiment (5.5 days) did not express detectable levels of GAP-43, indicating that the expression of this gene is due to addition of BDNF.

The PI 3-K inhibitor LY294002 blocks BDNF-mediated survival

PI 3-K is a heterodimer composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit that phosphorylates phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate on the D3 position of the inositol ring, leading to the formation of phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate, respectively (Kapeller and Cantley, 1994). In PC12 cells these lipids have been shown to activate the Akt/PKB pathway after NGF stimulation (reviewed by Marte and Downward, 1997). PI 3-K and Akt have been involved in the survival-promoting effect of NGF (Yao and Cooper, 1995) and other growth factors (Yao and Cooper, 1996; D'Mello et al., 1997; Dudek et al., 1997; Miller et al., 1997). Cells pretreated for 5 days with 10 μM RA were switched to serum-free medium containing BDNF (2 n*M*) plus increasing concentrations (1, 5, and 10 μM) of LY294002, a specific inhibitor of PI 3-K (Vlahos et al., 1994). After 48 h in culture, cell survival was evaluated. LY294002 reduced the number of surviving cells in a dose-dependent manner when compared with cultures treated with BDNF alone (Fig. 7A).

When the state of phosphorylation of Akt was monitored in cultures treated with BDNF or BDNF plus different concentrations of LY294002, a clear dose-dependent decrease in Akt phosphorylation was observed for increasing concentrations (1, 5, and 10 μM) of the drug. At 10 μM, the Akt phosphorylation was inhibited to values similar to the ones observed for nonstimulated controls (Fig. 7B). Reprobing of the membrane with an anti-pan-Akt antibody showed that protein loading between the different lanes was similar (Fig. 7B). To elucidate whether these changes in Akt phosphorylation correlated with a decrease in PI 3-K activity, we measured the generation of phosphatidylinositol 3-phosphate from L-α-phosphatidylinositol and L-α-phosphatidylserine precursors on anti-phosphotyrosine immunoprecipitates. Increasing concentrations of LY294002 caused a gradual decrease in the amount of phosphatidylinositol 3-phosphate generated (Fig. 7B), thus revealing a strong correlation between PI 3-K activity and phosphorylation of Akt.

FIG. 3. Effects of neurotrophins on RA-pretreated SH-SY5Y cells. **A:** Cells were treated for 5 days with 10 μM RA and then cultured in serum-free medium containing no additives (NA) or the indicated neurotrophin (2 n*M*). After an additional period of 48 h, cell survival was assessed by trypan blue staining. Data are mean ± SEM values. **B:** Cells were treated in a similar way as in A, and after 24 h in the presence of the corresponding neurotrophin, neurite outgrowth was scored as described in Materials and Methods. $^*p < 0.05$ in A and B indicates significant differences between the indicated treatments and the control condition (NA) by the unpaired, two-tailed Student's *t* test. **C:** Representative fields of each condition established in B were photographed under a phase-contrast microscope.

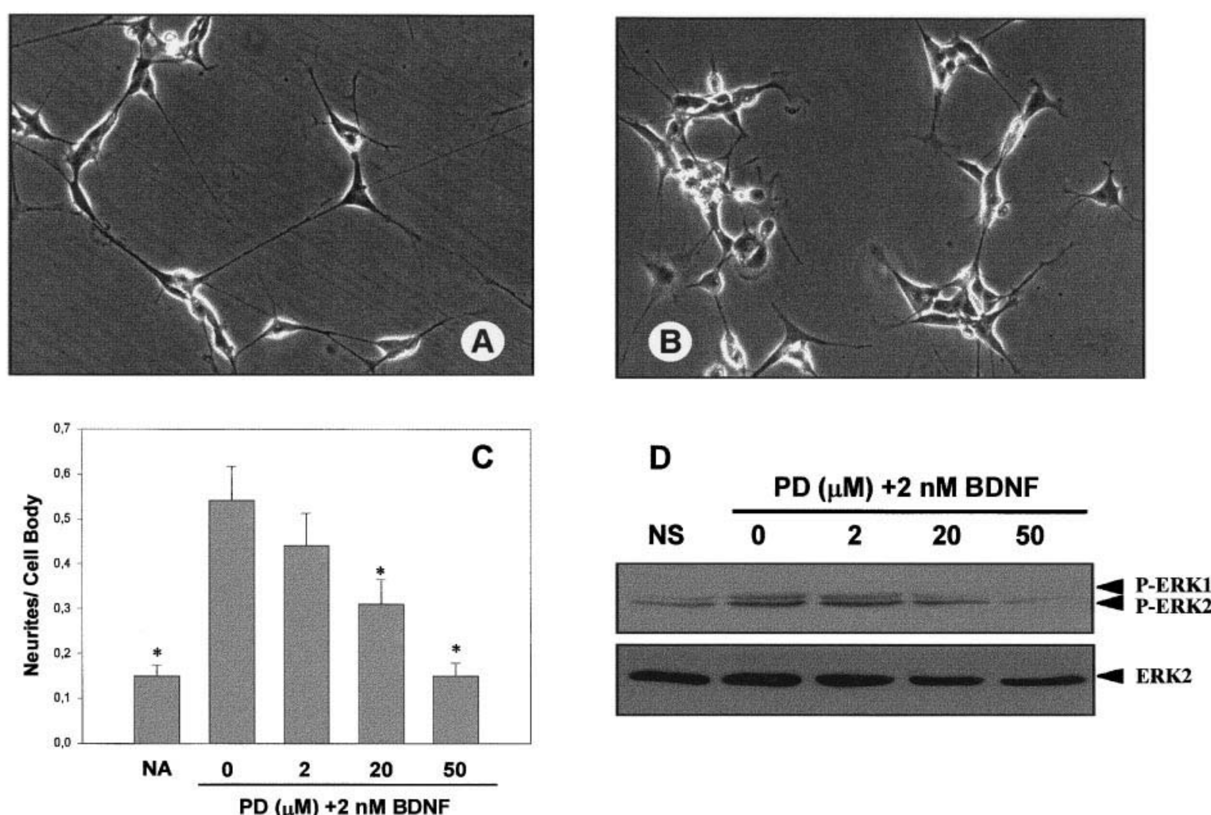


FIG. 5. Effects of PD98059 (PD) on BDNF-mediated differentiation of RA-pretreated SH-SY5Y cells. Cells were exposed for 5 days to 10 μM RA and then switched to serum-free medium containing 2 n*M* BDNF plus different concentrations of PD for 24 h. **A** and **B:** Phase-contrast micrographs of cultures exposed to BDNF alone or BDNF plus 20 μM PD, respectively. **C:** Neurite outgrowth was scored by the ratio of the number of neurites:number of cells in serum-free medium containing no additives (NA) or 2 n*M* BDNF plus different concentrations of PD. $^*p < 0.05$ indicates significant differences between the asterisk-labeled condition and BDNF without PD. Data are mean ± SEM (bars) values. **D:** Effect of PD on the activation of ERK1 and 2 MAPKs measured as the degree of tyrosine kinase phosphorylation. Cells were maintained in 10 μM RA for 5 days, preincubated for 30 min with the indicated doses of PD, and then stimulated with 2 n*M* BDNF for 5 min. Tyrosine phosphorylation of ERKs was assessed by western blotting with specific anti-phospho-ERK antibody (**top**). Reprobing the filter with anti-pan-ERK was used as a control of gel loading (**bottom**). NS, nonstimulated control cells.

LY294002 causes cell death with morphological features of apoptosis

To analyze the morphological features of the cell death induced by LY294002, cells were stained with the DNA dye Hoechst 33258. LY294002 caused cells to exhibit condensed and fragmented chromatin characteristic of apoptosis. These morphological features were also present in cells cultured in a medium without additives (Fig. 8).

When the number of apoptotic nuclei was quantified, no significant effects of LY294002 were found at 1 μM compared with BDNF alone. However, the number of apoptotic nuclei progressively increased with 5 and 10 μM LY294002. At 10 μM, the amount of apoptotic nuclei was similar to that obtained for cells cultured in basal medium, i.e., without either BDNF or LY294002 (Fig. 8).

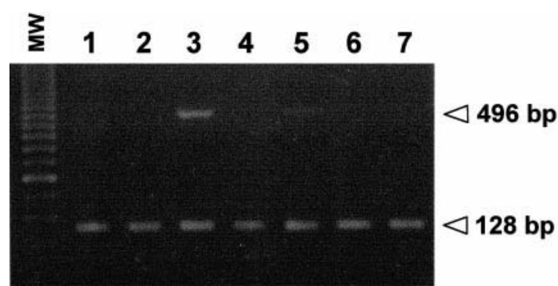


FIG. 6. Agarose gel shows the RT-PCR product for GAP-43 (**upper**) coamplified with the control protein L27 (**lower**). Cells were treated for 5 days with 10 μM RA and then were cultured in serum-free medium containing 2 n*M* BDNF alone (lane 3, 6 h of incubation; lane 5, 12 h of incubation) or BDNF plus 20 μM PD98059 (lane 4, 6 h of incubation, lane 6, 12 h of incubation). Lanes 2 and 7 correspond to cells treated for 5 and 5.5 days with RA, respectively, whereas lane 1 corresponds to naive cells, i.e., cells not pretreated with RA. The same results were obtained without L27 amplification. MW, 50-bp Molecular Size Marker ladder (Pharmacia).

DISCUSSION

In this study we have examined the biological effects of neurotrophins in SH-SY5Y cells induced to present

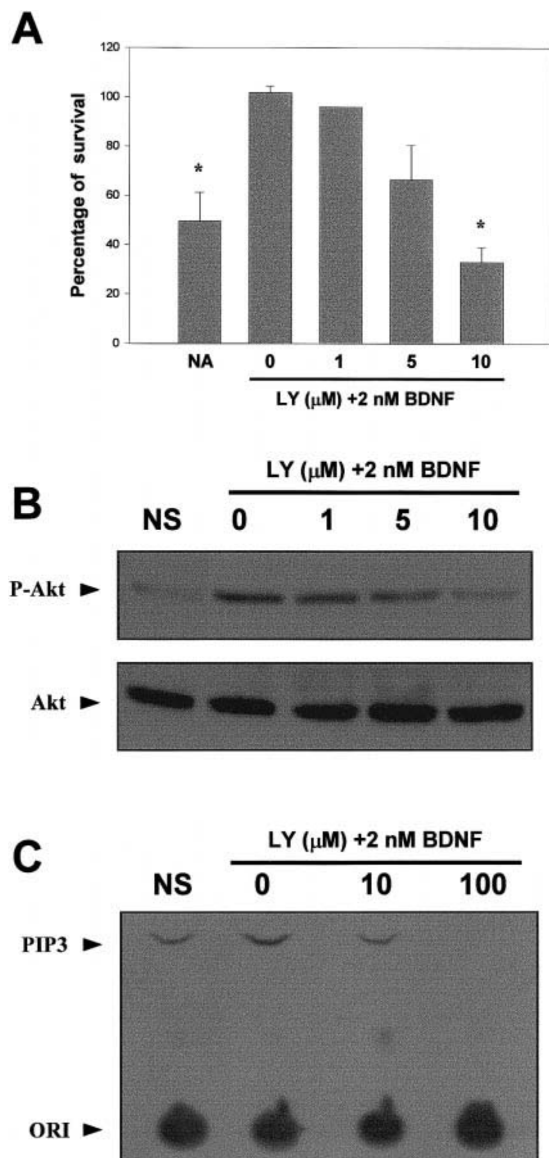
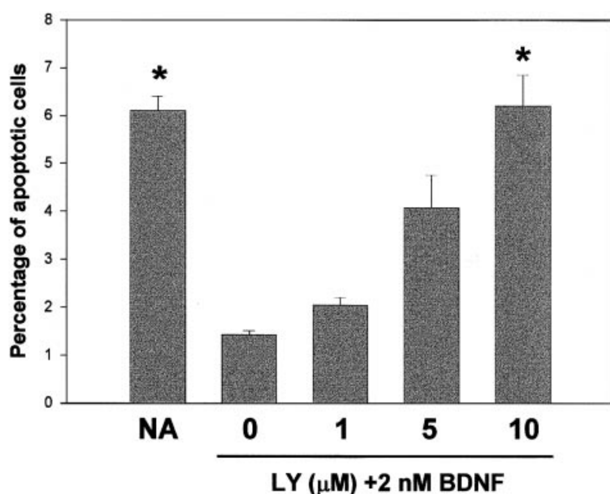


FIG. 7. A: Effects of LY294002 (LY) on BDNF-mediated survival of RA-pretreated SH-SY5Y cells. Cells were exposed for 5 days to 10 *μM* RA and then switched to serum-free medium containing 2 n*M* BDNF plus different doses of LY for an additional period of 48 h. $^{*}p < 0.05$ indicates significant differences between the asterisk-labeled condition and BDNF without PD98059. Data are mean ± SEM (bars) values. **B:** Effects of LY on Akt phosphorylation. Cells were exposed to 10 *μM* RA for 5 days and preincubated with the indicated doses of the drug for 30 min. Stimulation with 2 n*M* BDNF was performed, and the state of Akt phosphorylation was assessed by western blot using a specific anti-phospho-Akt antibody (**top**). The filter was reprobed with an anti-pan-Akt antibody to control the protein content per lane (**bottom**). NS, nonstimulated cells. **C:** Effects of LY on PI 3-K activity. Cells treated and stimulated as in A were lysed, and anti-phosphotyrosine immunoprecipitates were obtained. The PI 3-K activity was measured as described in Materials and Methods, including the indicated doses of LY in the reaction mixture. The arrowheads indicate the point of application of the samples (ORI) and the position of the phosphatidylinositol 3-phosphate (PIP3) standard.

high levels of functional Trk B, Trk C, and, to a much lesser extent, Trk A receptors on their surface. Previous studies have demonstrated that SH-SY5Y cells express high levels of Trk B after exposure to RA. These receptors undergo autophosphorylation when stimulated with BDNF, NT-3, or NT-4/5 (Kaplan et al., 1993). In agreement with these data, we have detected the appearance of functional Trk receptors in these cells over a period of 7 days of RA treatment. NGF-induced Trk phosphorylation was very weak when compared with BDNF, NT-3, and NT-4/5, which strongly stimulated Trk autophosphorylation. NT-3 was able to induce Trk autophosphorylation at doses as low as 200 p*M,* suggesting that Trk C is present on the surface of these cells. NT-3 has been reported to stimulate with similar affinity Trk B and Trk C in nonneuronal systems such as fibroblasts. In contrast, in a neuronal context, the affinity of NT-3 for Trk B decreases >100-fold (Ip et al., 1993). However, recent data indicate that NT-3 is able to mediate neuronal survival responses in vivo through a direct interaction with Trk B (Fariñas et al., 1998). In our system, NT-3 seems to be acting through Trk C rather than through Trk B or Trk A, because only the Trk C-specific antibody is able to immunoprecipitate the Trk phosphorylated by NT-3.

Our data show that neurotrophins support cell survival under serum-free conditions to very different extents. BDNF and NT-4/5 exerted the most striking effects on cell survival. The differences in the number of viable cells between cultures containing BDNF compared with those lacking trophic support are not due to a proliferative effect of this neurotrophin, because the values for survival are similar (~90%) to the number of initial cells, i.e., 5 days of RA treatment. Moreover, it has been reported that BDNF does not alter [^{3}H]thymidine uptake in RA-pretreated SH-SY5Y cells (Matsumoto et al., 1995). On the other hand, NT-3 showed a moderate effect on cell survival, with this value being significantly lower than that observed with BDNF or NT-4/5. Several authors have found that BDNF or NT-4/5 but not NGF or NT-3 could promote neuronal survival in primary cultures of rat cerebellar granule cells, although they express both Trk B and Trk C (Segal and Greenberg, 1992; Lindholm et al., 1993; Gao et al., 1995; Kubo et al., 1995; Zirrgiebel et al., 1995; Nonomura et al., 1996; Zirrgiebel and Lindholm, 1996). However, in these cells differences in the phosphorylation of PLCγ have been reported. PLCγ is phosphorylated by BDNF or NT-4/5 but not by NT-3 (Nonomura et al., 1996; Zirrgiebel and Lindholm, 1996). When other intracellular signaling proteins were analyzed, including the receptor autophosphorylation, PI 3-K, ERKs, or c-fos, no clear differences could be found between NT-3 and BDNF or NT-4/5 (Lindholm et al., 1993; Zirrgiebel and Lindholm, 1995; Nonomura et al., 1996). In our system, the level of Trk C autophosphorylation induced by NT-3 was comparable to the Trk B phosphorylation induced by either BDNF or NT-4/5. Moreover, NT-3 induced ERKs and Akt phosphorylation to the same extent as BDNF or NT-4/5. Therefore, the limited ability of NT-3 to support neuro-



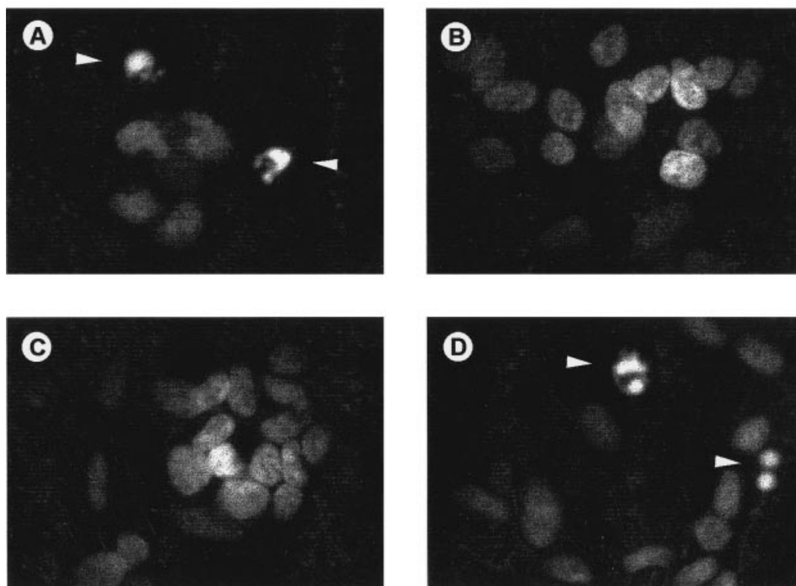


FIG. 8. LY294002 (LY) prevents the anti-apoptotic effect of BDNF. **Top:** Cells were exposed to 10 μM RA for 5 days and then switched to a serum-free medium containing no additives (NA), BDNF (2 nM), or BDNF plus the indicated doses of LY for an additional 48 h. Nuclei were stained with Hoechst 33258, and apoptotic cells were counted (top). $^{*}p < 0.05$ indicates significant differences between the asterisk-labeled condition and BDNF without LY by unpaired, two-tailed Student's t test. Data are mean ± SEM (bars) values. **Bottom:** Representative fields were photographed under UV illumination with a fluorescence microscope. **A:** No additives. **B:** BNDF. **C:** BDNF plus 1 μM LY. **D:** BDNF plus 10 μM LY. Arrowheads indicate apoptotic nuclei.

nal survival did not correlate with the level of activation of the signaling pathways triggered by Trk C that are probably involved in cell survival (see below). A possible explanation for this phenomenon could be a differential element in the transduction pathways initiated by BDNF and NT-3 such as PLCγ (Nonomura et al., 1996; Zirrgiebel and Lindholm, 1996). Another possibility could be differences in the transient or sustained activation of the intracellular pathways elicited by each neurotrophin. In any case, further studies are required to elucidate which is the molecular basis of this differential behavior.

Neuritic outgrowth was also affected in different ways by neurotrophins. We have found that BDNF and NT-4/5 can induce a profuse neuritogenic response on RA-treated SH-SY5Y cells. Moreover, GAP-43, a neuronal marker that has been widely used as an indicator of cell differentiation, is rapidly expressed after addition of BDNF (6 h). In agreement with these results it has been demonstrated that PC12 cells stably transfected with Trk B can differentiate after addition of BDNF (Iwasaki et al., 1997). On the other hand, NT-3-treated cells showed an intermediate phenotype, and NGF-treated cells display little if any change in neuritic outgrowth. Matsumoto et al. (1995) reported a similar behavior of these cells when exposed simultaneously to RA and each of these neurotrophins.

To date, little is known about the signaling events that are initiated by Trk B and Trk C, when compared with the more abundant information available for Trk A. NT-3 has been shown to induce phosphorylation of MAPK in oligodendrocytes (Cohen et al., 1996). In cultures of

cerebellar granule cells, BDNF, NT-3, and NT-4/5 triggered the phosphorylation of Shc, PI 3-K, and ERK/MAPKs, and the expression of c-fos, whereas only BDNF and NT-4/5 seemed to activate PLCγ (Lindholm et al., 1993; Zirrgiebel et al., 1995; Nonomura et al., 1996; Zirrgiebel and Lindholm, 1996). In hippocampal pyramidal neurons, BDNF and NT-3 induce various signal transduction events, such as activation of p21ras, MAPK, or PLCγ. However, none of these neurotrophins is able to support the survival of these neurons (Marsh and Palfrey, 1996). We show here that BDNF, NT-3, or NT-4/5 induces the phosphorylation of both ERKs and Akt. Moreover, we also show that at least BDNF is able to increase the PI 3-K activity in RA-treated cultures.

The main goal of this work was to elucidate the relative significance of the Ras/MAPK and PI 3-K/Akt pathways in neuritogenesis and survival. The role of the Ras/MAPK pathway in differentiation and survival is controversial (reviewed by Kaplan and Miller, 1997). Various reports, based in the PC12 cell line, which spontaneously expresses high levels of Trk A, have shown that a persistent activation of Ras/MAPK pathway is necessary for differentiation responses in this system. Transfection of oncogenic *ras* in PC12 cells leads to sustained ERK activation and neuronal differentiation (Qiu and Green, 1992), whereas the expression of dominant-negative MEK mutants blocks the growth factor-mediated differentiation of PC12 cells (Cowley et al., 1994). Moreover, the selective MEK inhibitor PD98059 blocks the differentiation of PC12 cells induced by NGF (Pang et al., 1995). However, Borasio et al. (1993) have demonstrated that the activity of Ras is neither necessary nor sufficient for NGF-mediated neuritogenesis of chick sympathetic neurons. In addition, PD98059 failed to block NGF-induced neurite outgrowth in chick sensory and sympathetic neurons (Klinz et al., 1996). Moreover, very little information exists regarding the intracellular pathways involved in neuritogenesis induced by BDNF through Trk B receptors. Our results suggest that the Ras/MAPK pathway is necessary for BDNF-mediated neuritogenesis in RA-pretreated SH-SY5Y cells. PD98059 blocked neuritic outgrowth in a dose-dependent manner, at a range of concentrations that effectively prevented the activation of ERK1 and ERK2 by BDNF. Moreover, the expression of GAP-43 was affected in the same way by this inhibitor. These findings are in agreement with those reported by Kim et al. (1997), who described the requirement of MAPK activation for neuritogenesis in SH-SY5Y cells using a related growth factor, insulin-like growth factor-I.

On the other hand, PD98059 failed to block the survival-promoting effect of BDNF, suggesting that MAPK activity is not needed for this effect. In agreement with this, MAPK activation is not necessary for survival of sympathetic neurons (Creedon et al., 1996; Virdee and Tolkovsky, 1996), hippocampal pyramidal neurons (Marsh and Palfrey, 1996), and chicken spinal motor neurons (Soler et al., 1998; Dolcet et al., 1999). Neutralizing Ras antibodies, however, block neuronal survival (Borasio et al., 1993; Nobes and Tolkovsky, 1995), suggesting that a neuronal survival pathway branches from the MAPK pathway at the level of Ras.

Increasing numbers of reports show the connection between neuronal survival and the activation of PI 3-K and Akt (Yao and Cooper, 1995; D'Mello et al., 1997; Dudek et al., 1997). We present evidence for an anti-apoptotic function of the PI 3-K/Akt pathway. Inhibition of PI 3-K by LY294002 in the presence of BDNF resulted in the reversal of the survival effect of this neurotrophin, at concentrations that reduced BDNF-induced PI 3-K activity and Akt phosphorylation to levels comparable to the one obtained in nonstimulated cells. In the absence of BDNF or when treated with BDNF plus LY294002, cells underwent an apoptotic cell death that was morphologically indistinguishable between both conditions.

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6.2. El tratamiento secuencial con ácido retinoico y BDNF da lugar a células con fenotipo neuronal dependientes de BDNF

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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

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Abstract: A rapid and simple procedure is presented to obtain nearly pure populations of human neuron-like cells from the SH-SY5Y neuroblastoma cell line. Sequential exposure of SH-SY5Y cells to retinoic acid and brain-derived neurotrophic factor in serum-free medium yields homogeneous populations of cells with neuronal morphology, avoiding the presence of other neural crest derivatives that would normally arise from those cells. Cells are withdrawn from the cell cycle, as shown by 5-bromo-2′-deoxyuridine uptake and retinoblastoma hypophosphorylation. Cell survival is dependent on the continuous presence of brain-derived neurotrophic factor, and removal of this neurotrophin causes apoptotic cell death accompanied by an attempt to reenter the cell cycle. Differentiated cells express neuronal markers, including neurofilaments, neuron-specific enolase, and growth-associated protein-43 as well as neuronal polarity markers such as tau and microtubule-associated protein 2. Moreover, differentiated cultures do not contain glial cells, as could be evidenced after the negative staining for glial fibrillary acidic protein. In conclusion, the protocol presented herein yields homogeneous populations of human neuronal differentiated cells that present many of the characteristics of primary cultures of neurons. This model may be useful to perform large-scale biochemical and molecular studies due to its susceptibility to genetic manipulation and the availability of an unlimited amount of cells. **Key Words:** Neuroblastoma—Neurotrophin—SH-SY5Y cells—Neurodegenerative disease—Cell cycle.
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In metazoan organisms, maintenance of homeostasis requires the proper relationships among cell proliferation, differentiation, and death. Somatic cells proliferate and divide by executing tightly regulated processes during the cell cycle, whereas apoptotic cell death allows an organism to eliminate unwanted cells through a safe, orderly process. In the case of the nervous system, roughly half of the neurons initially generated die by apoptosis in a well-defined interval, coincident with the establishment of synaptic connections. This cell death is known as naturally occurring or programmed cell death (PCD), and it is believed to match the number of innervating neurons to the size of the target cell population, as well as to eliminate aberrant synaptic contacts (Oppenheim, 1991; Henderson, 1996). Apoptotic neuronal cell death that occurs outside this developmental window may contribute to certain pathological conditions such as Alzheimer's disease (Anderson et al., 1996).

PCD seems to result from the failure of individual neurons to gain access to neurotrophic factors that are released in limited amounts by target tissues, glial cells, or afferent inputs (Burek and Oppenheim, 1996). The family of neurotrophins [composed mainly by nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3, and neurotrophin 4/5] is the

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Abbreviations used: BDNF, brain-derived neurotrophic factor; BrdU, 5-bromo-2′-deoxyuridine; cdk, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle's medium; GAP-43, growth-associated protein-43; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; NGF, nerve growth factor; NF-L, -M, and -H, low-, medium-, and high-molecular-weight neurofilaments, respectively; NSE, neuron-specific enolase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, PCD, programmed cell death; pRB, retinoblastoma susceptibility gene product; RA, retinoic acid; SDS, sodium dodecyl sulfate; Trk, tyrosine receptor kinase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

best-characterized group of such neurotrophic factors and has been shown to promote survival of several types of neurons both in vivo and in vitro. Targeted disruption of the genes encoding for neurotrophins or their specific tyrosine kinase receptors (collectively known as Trks) causes cell loss of specific neuronal populations (reviewed by Klein, 1994; Snider, 1994). When cultured in vitro, different neuronal populations show a selective survival response to particular neurotrophins, and their removal triggers an apoptotic cell death that mimics the phenomenon of PCD (reviewed by Davies, 1994; Lewin and Barde, 1996).

The molecular mechanisms underlying the regulation of the apoptotic process are objects of intense study. Recent lines of evidence argue for a strong interrelationship between cell cycle control and apoptosis. These findings include morphological changes occurring during apoptosis that are reminiscent of mitosis, as well as involvement of cell cycle-regulatory molecules such as p53, retinoblastoma susceptibility gene product (pRB), E2F, and cyclin-dependent kinases (cdks) in the apoptotic process (reviewed by Kasten and Giordano, 1998; King and Cidlowski, 1998). In the nervous system, several cell cycle-regulatory molecules are expressed, including cyclins and cdk4 and 5, although neurons are postmitotic cells (Freeman et al., 1994). More important is that, in sympathetic neurons, NGF withdrawal induces a selective increase in the levels of cyclin D1 (Freeman et al., 1994), and overexpression of this molecule in N1E-115 cells leads to apoptotic cell death (Kranenburg et al., 1996). In staggerer and lurcher mutant mice, which show massive death of cerebellar granule cells due to defects in the development of their synaptic targets, an elevation of cyclin D and proliferating cell nuclear antigen levels and 5-bromo-2′-deoxyuridine (BrdU) uptake is observed during the onset of cell death (Herrup and Busser, 1995). Moreover, chemical inhibitors of cdks or dominant-negative forms of cdk4 and cdk6 promote survival of NGF-deprived sympathetic neurons (Park et al., 1997) and KCl-deprived cerebellar granule cells (Padmanabhan et al., 1999). These and other observations had led to the hypothesis that apoptosis occurs after an attempt to enter the S-phase of the cell cycle in an inadequate cellular context, where contradictory or conflicting growth signals may converge. Finally, alterations in the expression of cell cycle-related proteins have been described in neurodegenerative disorders such as Alzheimer's disease (Vincent et al., 1997; Nagy et al., 1997; Busser et al., 1998; Giovanni et al., 1999) and other pathological situations (Nagy and Esiri, 1998; Timsit et al., 1999).

Much of the above information has been obtained by the use of primary cultures of neurons. However, the reduced amount of cells obtained and the limited susceptibility of genetic manipulation of these systems make biochemical and molecular approaches difficult to perform. Another important handicap is the ethical problem that arises when using human embryonic neurons for pathophysiological studies of human neurodegenerative diseases. Several protocols that yield neuronal differentiated cells have been applied to human cell lines (see, for example, Pleasure et al., 1992; Hill and Robertson, 1997; Prince and Oreland, 1997). However, most of them are time-consuming and do not yield a population with known trophic dependencies, a requirement that must be accomplished to mimic the phenomenon of PCD that occurs in vivo and in primary cultures of neurons. In this work, we present a protocol of differentiation based on the sequential treatment of the SH-SY5Y human neuroblastoma cell line with retinoic acid (RA) and BDNF. This protocol yields homogeneous populations of neuronal differentiated cells that are strictly dependent on BDNF for their survival. When BDNF is removed from the culture medium, cells enter an apoptotic cell death accompanied by an attempt to reenter the cell cycle. This model seems to mimic naturally occurring cell death and could be a useful tool to perform large-scale biochemical and genetic studies.

MATERIALS AND METHODS

Cell culture

The SH-SY5Y neuroblastoma cell line was kindly provided by Dr. D. Martin-Zanca (Salamanca, Spain). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 m*M* L-glutamine, penicillin (20 units/ml), streptomycin (20 mg/ml), and 15% (vol/vol) heat-inactivated fetal calf serum (GIBCO, Gaithersburg, MD, U.S.A.). Cells were maintained at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO_2. Cells were seeded at an initial density of 10^4 cells/cm^2 in culture dishes (Corning, Corning, NY, U.S.A.) previously coated with 0.05 mg/ml collagen (Collaborative Biomedical Products, Bedford, MA, U.S.A.). *all-trans*-RA (Tocris Cookson, Bristol, U.K.) was added the day after plating at a final concentration of 10 μM in DMEM with 15% fetal calf serum. After 5 days in the presence of RA, cells were washed three times with DMEM and incubated with 50 ng/ml BDNF (Alomone Laboratories, Jerusalem, Israel) in DMEM (without serum) for different intervals.

Immunoprecipitation and western blot

Immunoprecipitation of Trk receptors was performed as previously described (Encinas et al., 1999). For western blot experiments, cells were washed with phosphate-buffered saline (PBS), lysed with 2% sodium dodecyl sulfate (SDS) and 125 m*M* Tris (pH 6.8), sonicated, and boiled for 5 min. Protein was quantified by means of the Bio-Rad DC protein assay. Protein (20–50 μg) was resolved in standard SDS-polyacrylamide gel electrophoresis (PAGE) minigels (Miniprotean; Bio-Rad) and were transferred to an Immobilon-P membrane (Millipore, Bedford) using a Pharmacia semidry Transblot. The SDS-PAGE conditions for pRB were slightly modified. Thus, pRB hyperphosphorylated forms were resolved in 6% acrylamide gels (see Fig. 4C, left panel), whereas the cleaved pRB product was identified in long (20-cm) 7.5% acrylamide gels (see Fig. 4C, right panel). Membranes were blocked in Tris-buffered saline with Tween 20 [20 m*M* Tris (pH 7.4), 150 m*M* NaCl, and 0.05% Tween 20] containing 5% nonfat dry milk for 30 min at room temperature. Primary antibodies were incubated for 1 h at room temperature and subsequently incubated with peroxidase-conjugated antibodies. Blots were finally developed with the enhanced chemiluminescence western blotting detection sys-

tem (Amersham, Little Chalfont, Bucks, U.K.). The following antibodies were used at the dilutions recommended by the manufacturers: α-neuron-specific enolase (NSE; Cambridge Research Biochemicals, Cheshire, U.K.), α-growth-associated protein-43 (GAP-43; Transduction Laboratories, Lexington, KY, U.S.A.), α-glial fibrillary acidic protein (GFAP), α-low-molecular-weight neurofilament (NF-L; 68K), and α-medium-molecular-weight neurofilament (NF-M; 160K) (Sigma, Madrid, Spain), and α-pRB (PharMingen, San Diego, CA, U.S.A.).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay

Cells were fixed in freshly prepared 2% paraformaldehyde for 30 min at room temperature and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 10 min at 4°C. Subsequently, cells were incubated with 50 μl of a reaction mixture containing 0.3 nmol of fluorescein-12-UTP (Boehringer Mannheim, Barcelona, Spain), 3 nmol of dATP, and 20 units of terminal deoxynucleotidyl transferase (GIBCO) for 1 h at 37°C in a humidified chamber. Cells were then washed with PBS and incubated with 0.05 μg/ml Hoechst 33258 (Sigma) for 30 min at room temperature. Finally, cells were mounted with Fluoprep (BioMérieux, Marcy l'Etoile, France) and observed under UV illumination in an epifluorescence microscope (Nikon, ECLIPSE model 600; Izasa, Barcelona) coupled to a digital CCD camera (AstroCam; Life Sciences Resources, Cambridge, U.K.). Image analysis was performed with Esprit Rego software (Life Sciences Resources). Quantitative data were obtained by scoring at least 500 cells under each experimental condition in three replicas. Experiments were repeated at least two times, and counts were made in a blinded manner.

Caspase activity

Cells were rinsed with ice-cold PBS and lysed in a buffer containing 100 m*M* HEPES (pH 7.4), 5 m*M* dithiothreitol, 5 m*M* EGTA, 0.04% NP-40, and 20% glycerol. Extracts were then centrifuged at 5,000 *g* for 10 min, and protein concentrations were determined by the assay of Bradford (1976). Cell extracts (10–20 μg) were diluted in 50 μl of reaction buffer [100 m*M* HEPES (pH 7.4), 5 m*M* dithiothreitol, 5 m*M* EGTA, 0.04% NP-40, and 20% glycerol] and incubated with 100 μ*M* fluorescent substrate *N*-benzyloxycarbonyl-DEVD-7-amino-4-trifluoromethylcoumarin (Enzyme System Products, Livermore, CA, U.S.A.) at 37°C for 1 h. The fluorescent signals were determined with a spectrofluorometer (Bio-Tek Instruments, Winooski, VT, U.S.A.) at an excitation wavelength of 360 nm and an emission wavelength of 530 nm. Protease activity was expressed as the amount of cleaved substrate (7-amino-4-trifluoromethylcoumarin) per microgram of protein.

BrdU incorporation and cell cycle analysis

Cells were incubated with 20 μ*M* BrdU (Sigma) for 4 h at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO_2. Subsequently, cells were fixed in 2% paraformaldehyde for 30 min and subjected to immunodetection using a fluorescein-conjugated anti-BrdU antibody from the In Situ Cell Proliferation Kit FLUOS (Boehringer Mannheim) following the manufacturer's instructions. To analyze the cell cycle distribution, cells were counterstained with 50 μg/ml propidium iodide and 20 μg/ml RNase A on ice for 15 min. FACS analysis was performed with an EPICS XL flow cytometer (Coulter).

Immunocytofluorescence of heavy-molecular-weight neurofilament (NF-H), microtubule-associated protein 2 (MAP2), and tau

Cells were fixed in 4% paraformaldehyde in PBS at room temperature for 20 min, washed three times with PBS containing 0.1% Triton X-100, and blocked for 40 min with PBS containing 1% bovine serum albumin and 0.1% Triton X-100. They were then incubated overnight with monoclonal α-NF-H (1:40; 200K; Sigma), α-MAP2 (1:50; Sigma), or α-Tau-1 (1:50; Boehringer Mannheim) antibodies diluted in PBS containing 1% bovine serum albumin and 0.1% Triton X-100, washed three times in PBS with 0.1% Triton X-100, and incubated at room temperature for 40 min with fluorescein isothiocyanate-conjugated donkey anti-mouse monoclonal antibody (1:250 in PBS with 0.1% Triton X-100; Jackson Immunoresearch, West Grove, PA, U.S.A.). Finally, they were washed three times with PBS and mounted with Mowiol (Calbiochem-Novabiochem, La Jolla, CA, U.S.A.) plus 0.1% 1,4-diazabicyclo[2.2.2]octane (Sigma) as an antifading agent. The observation was carried out in an epifluorescence microscope (see above).

Measurement of neurotransmitter content and noradrenaline release

The neurotransmitter content of the untreated cells and cells treated with RA for 5 days or RA for 5 days plus BDNF for 7 days was assayed by quantitative HPLC according to the procedure of Calvo et al. (1995).

For noradrenaline release assays, cells were seeded in 24-well dishes and either left untreated or treated with RA for 5 days or with RA for 5 days followed by BDNF for 7 days. Cells were then rinsed twice with HEPES-buffered saline (135 m*M* NaCl, 5 m*M* KCl, 0.6 m*M* $MgSO_4$, 2.5 m*M* $CaCl_2$, 10 m*M* HEPES, and 6 m*M* glucose) containing 20 μg/ml pargyline and 20 μg/ml ascorbic acid (pH 7.4) and loaded with 50 n*M* [^{3}H]noradrenaline for 1 h at 37°C. After washing off the excess of noradrenaline, cells were treated with 10 n*M* 12-*O*-tetradecanoylphorbol 13-acetate (TPA) for 8 min immediately before evoking the release with 1 m*M* carbachol. Unreleased noradrenaline was extracted with 0.4 *M* perchloric acid, and the release was calculated as a percentage of the total amount of radioactivity present before evoking the release. Data are mean ± SEM values of three independent experiments (n = 4 for each condition), for which basal release has been subtracted.

RESULTS

Effects of long-term RA treatment in SH-SY5Y cells

The SH-SY5Y cell line is a third successive subclone of the SK-N-SH line, originally established from a bone marrow biopsy of a neuroblastoma patient (Biedler et al., 1973). The SK-N-SH parental line comprises at least two morphologically and biochemically distinct phenotypes: neuroblastic (N-type) and substrate adherent (S-type), which can undergo transdifferentiation (Ross et al., 1983). Although derived from a neuroblastic subclone, the SH-SY5Y line retains a low proportion of S-type cells (Fig. 1A, arrowheads). In agreement with previous reports (Pahlman et al., 1984), when cultures were treated with 10 μ*M* RA for 5 days in complete medium (DMEM plus 15% fetal calf serum), a considerable proportion of neuroblastic (N-type) cells differentiated to a

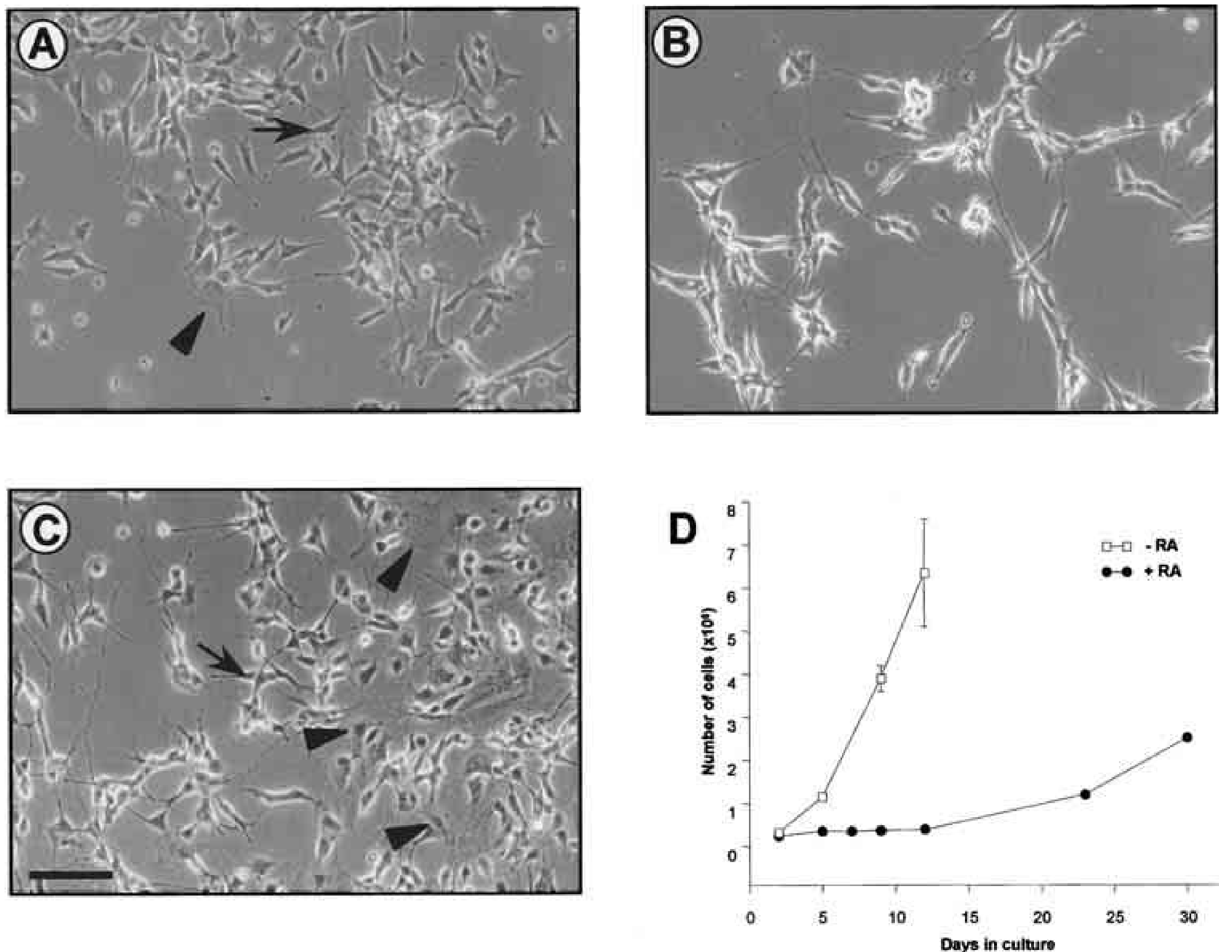

FIG. 1. Effects of long-term RA treatment in SH-SY5Y cells shown in phase-contrast micrographs of SH-SY5Y cells growing in complete medium (DMEM with 15% fetal calf serum) without additives (**A**) and in complete medium containing 10 μM RA for (**B**) 5 or (**C**) 10 days. Note that untreated cells are mainly neuroblastic (N-type, arrows), but occasionally some substrate-adherent cells appear (S-type, arrowheads). Treatment with RA resulted initially in neurite outgrowth, but as time in culture increased, S-type cells progressively overgrew the cultures. Bar = 50 μm. **D:** Growth rate of SH-SY5Y cells in the presence or absence of RA. The cell number was calculated by detaching cells at the indicated time points and counting them in a hemocytometer. Data are mean ± SEM (bars) values of three independent experiments.

more neuronal phenotype by extending neuritic processes (Fig. 1B), whereas S-type cells did not undergo apparent morphological changes. Moreover, after ~10 days of culture in the presence of RA, the percentage of S-type cells progressively increased (Fig. 1C), overgrowing the cultures in the subsequent days. To quantify the effects of RA in the cell proliferation rate, a growth curve was generated in the presence or absence of RA (Fig. 1D). In agreement with previous reports (Pahlman et al., 1984), RA inhibited the growth rate of SH-SY5Y cells during the first 8–10 days of treatment. However, longer periods of incubation with RA progressively increased the total number of cells owing to the accumulation of S-type cells. Thus, although short RA treatments were able to inhibit cell proliferation and induced a modest degree of neuronal differentiation, long-term treatments with this drug did not yield a homogeneously neuronal differentiated population. Actually, long-term RA treatments seemed to unbalance the proportion between the N-type and S-type phenotypes toward the S-type one.

Effects of BDNF and serum in RA-pretreated cells

The side effects described above make long-term RA treatment of SH-SY5Y unsuitable for the acquisition of homogeneous populations of differentiated cells with neuronal characteristics. However, it has been reported that RA induces the expression of TrkB in SH-SY5Y cells, making them responsive to BDNF (Kaplan et al., 1993). Moreover, it has also been shown that BDNF enhances the differentiating effects of RA (Arcangeli et al., 1999). We first tested whether in our experimental conditions RA was able to induce the expression of TrkB. As shown in Fig. 2A, after 3 days of RA exposure, the levels of functional TrkB present on the cell surface, i.e., the amount of Trk capable of undergoing BDNF-induced autophosphorylation, were significantly in-

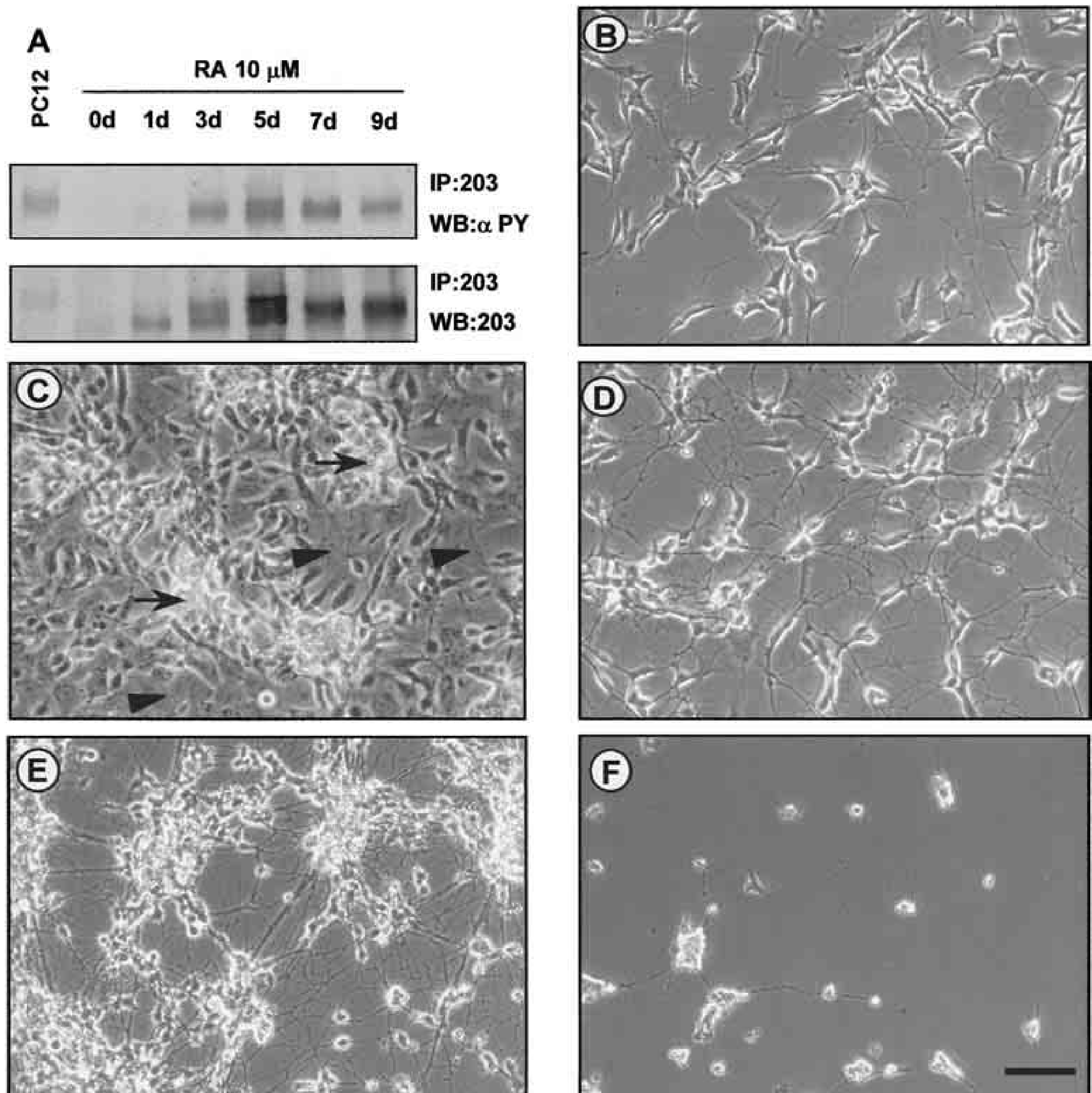

FIG. 2. Effects of serum and BDNF in RA-pretreated SH-SY5Y cells. **A:** Expression of TrkB after exposure of SH-SY5Y cells to RA. Cells were treated with 10 μM RA for the indicated times, stimulated with BDNF for 5 min, and immunoprecipitated (IP) with an anti-pan-Trk antiserum (203). The levels of active TrkB were detected by western blot (WB) with anti-phosphotyrosine antibody (**top panel**). The membrane was reincubated with antiserum 203 to visualize the amount of total Trk (**bottom panel**). Note that the highest levels of TrkB were reached at day 5 of RA. The PC12 lane corresponds to PC12 cells stimulated with NGF. **B:** After 5 days of treatment with RA, this agent was washed out from the medium, and BDNF (50 ng/ml) was added to analyze the biological effects elicited by TrkB. After 24 h of exposure to BDNF, cells showed a somewhat higher level of differentiation with more abundant and branched neurites. **C:** However, after 3 additional days under these conditions, S-type cells (arrowheads) overgrew the culture, and N-type cells (arrows) tend to aggregate. **D:** This could be circumvented by removing the serum from the culture medium at the time of addition of BDNF. In these conditions, virtually all the cells displayed a fully mature neuronal morphology, with round, phase-bright bodies and a robust network of neuritic processes connecting them. **E:** To assess the stability of the cultures treated with this protocol of differentiation, cells were pretreated 5 days with 10 μM RA and then incubated for 7 additional days in the presence of BDNF in serum-free medium. At this time point, there was no evidence of cellular degeneration, and S-type cells were not detectable. Moreover, cells remained in a differentiated state and showed a tendency to aggregate. **F:** In contrast, when in parallel cultures BDNF was not added to the medium after the removal of RA and serum, most cells retracted their neurites and died. Bar = 50 μm.

creased with respect to untreated cells. The levels of TrkB were maximal at day 5 of treatment and slightly decreased in the subsequent days. Accordingly, we decided to remove RA from the culture medium at day 5 of treatment and then switch the cultures to BDNF in complete medium.

After the first day of incubation with BDNF, cells scattered over the culture plate and displayed neuritic processes, with most of them displaying growth cones typical of primary cultures (Fig. 2B). However, with increasing culture time, S-type cells gradually appeared, forming a continuous layer below the N-type cells, which remained as small aggregates of rounded, refringent cells (Fig. 2C). In contrast, in parallel cultures where serum was removed at the time of addition of BDNF, a homogeneous population of cells with neuronal morphology was obtained, with a very low amount of S-type cells (Fig. 2D). In these cultures, cells showed rounded, phase-bright bodies and a profuse neuritic arborization forming extensive networks over the culture dish surface. Under these conditions, cultures were stable for at least 3 weeks, showing neither signs of cellular degeneration nor reversion of the neuronal phenotype. Under these conditions S-type cells were almost undetectable.

Dependence on BDNF for survival: neurotrophic deprivation induces apoptotic cell death

In a previous work, we reported that BDNF promoted short-term survival of RA-pretreated SH-SY5Y cells (Encinas et al., 1999). We wanted to characterize further whether this dependence on BDNF for survival persisted in long-term BDNF-treated cultures. Figure 2E shows a phase-contrast micrograph of cells treated for 5 days with RA and 7 additional days in the presence of BDNF in serum-free medium. Cells appeared healthy and showed a high degree of differentiation. In contrast, when parallel cultures were left in serum-free medium alone for the same interval, only a few cells with retracted neurites persisted (Fig. 2F). When cultures were maintained for 7 days in the presence of BDNF and then deprived for 7 additional days, the vast majority of cells degenerated and died (data not shown). Thus, at least for the intervals examined, BDNF seemed to be necessary for the survival of these cells.

Because in primary cultures removal of trophic agents leads to apoptotic cell death, we were interested in analyzing the characteristics of the cell death after BDNF deprivation. DNA fragmentation, a typical feature of apoptotic cell death, was assessed by means of the TUNEL reaction. Cultures treated for 5 days with RA were switched to either BDNF-containing or serum-free medium for 24 h. When BDNF was present, positive cells only occasionally appeared (Fig. 3C and D), whereas in cultures deprived of BDNF for the same interval, a significant number of labeled cells was found (Fig. 3A and B). It is noteworthy that chromatin condensation, another characteristic of apoptosis, was only found in cells that had been deprived of BDNF (Fig. 3B, arrowheads). The time course of cell death was quantified by scoring positive nuclei with respect to total nuclei stained with Hoechst 33258, and a representative graph is shown in Fig. 3E. Thus, morphological criteria indicated that the cell death followed by BDNF removal was apoptotic.

We also used biochemical approaches such as analysis of caspase activity to define this cell death as apoptotic. Caspases are cysteine proteases that cleave specific proteins in aspartic residues. Activation of these proteases accompanies the apoptotic process (for review, see Cryns and Yuan, 1998). Using a fluorogenic assay we observed a progressive increase in the caspase activity when cells treated for 5 days with RA were switched to serum-free medium. After 6 h of starvation, a detectable level of caspase activity above the background, i.e., the reaction mixture incubated without cell lysates, was measured, which increased until 24 h. When parallel cultures treated for 24 h in the presence of BDNF were subjected to such analysis, the amount of caspase activity was found to be about half of the one found in deprived cells (Fig. 3F). Taken together, these data strongly suggest that the cell death followed by BDNF withdrawal is apoptotic.

RA-BDNF-treated cells are arrested in G1

To analyze the cell cycle distribution during RA-BDNF treatment, we performed BrdU labeling experiments at different time points of the differentiation protocol. Cells were pulse-labeled for 4 h with BrdU before collecting and processing them for analysis with anti-BrdU fluorescein isothiocyanate-conjugated antibodies by means of flow cytometry. Propidium iodide staining was used to examine cell cycle distribution. Untreated cells displayed a typical profile of an asynchronously growing population, with ~30% of the cells traversing S-phase. Accordingly, elevated numbers of cells were BrdU-positive (Fig. 4A and B). After 5 days of RA treatment, there was a significant depletion of cells in the S and G2/M phases of the cell cycle, concomitant with a decrease in the number of BrdU-positive cells (Fig. 4A and B). This agrees with the observed stabilization in total cell numbers after RA treatment depicted in Fig. 1D. However, after this period, a small, although significant, population of cells was still capable of incorporating BrdU. When cells were examined after 3 days of exposure to BDNF, the percentage of BrdU-positive cells was <5%, and ~90% of cells were arrested in G1 (Fig. 4A and B). In conclusion, the RA-BDNF protocol yielded, as early as after 3 days in the presence of BDNF, a nearly pure population of cells arrested in the G1 phase of the cell cycle.

Withdrawal of BDNF induces S-phase entry and apoptotic cell death

When BDNF was withdrawn from the cultures, a subG1 peak typical of apoptotic cells was observed (Fig. 4A and B). A more detailed analysis showed that virtually all of these cells were present in the nonadherent fraction of cells, i.e., the cells that have died

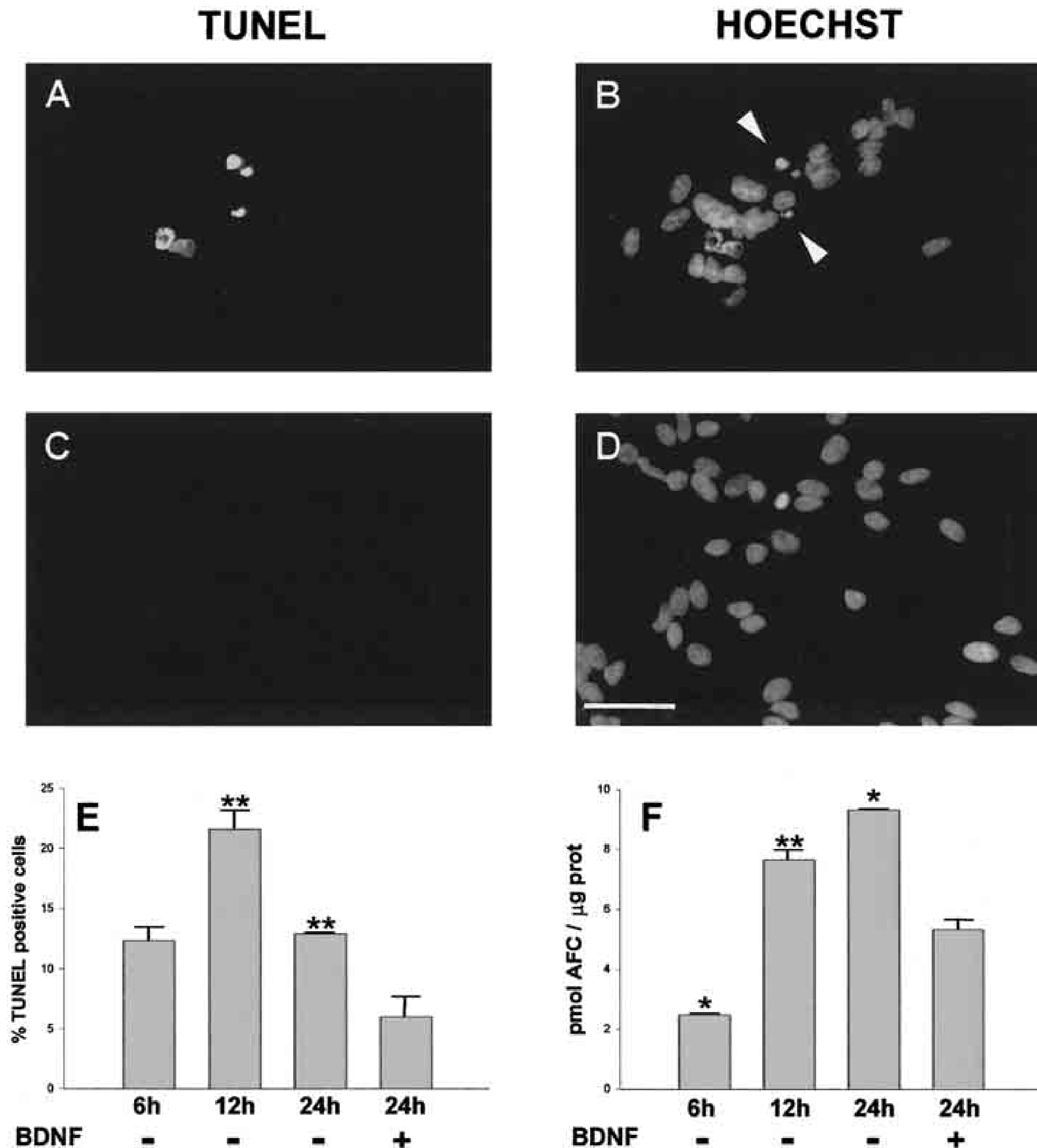

FIG. 3. BDNF-deprived SH-SY5Y cells are TUNEL-positive and have increased DEVD-directed caspase activity. SH-SY5Y cells treated for 5 days with RA and then left in serum-free medium alone (**A**) or supplemented with BDNF (**C**) for an additional 24 h were examined by TUNEL staining. **B** and **D:** Corresponding micrographs show the same microscopic fields with nuclei stained with the DNA dye Hoechst 33258. Note that in some TUNEL-positive cells (arrowheads) the chromatin is condensed in a typical apoptotic pattern. **E:** Time course of the cell death triggered by BDNF withdrawal. Cells were treated for 5 days with RA and then left for the indicated times in the absence (−) or presence (+) of BDNF. The percentage of TUNEL-positive cells was scored by counting at least 500 cells in each condition. **F:** DEVD-specific caspases are activated after BDNF removal. Cells were treated as in E, and the amount of 7-amino-4-trifluoromethylcoumarin (AFC) produced was measured in a fluorometric assay. Data are mean ± SEM (bars) values of two independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, by unpaired, two-tailed Student's *t* test with respect to BDNF treatment. Bar = 50 μm.

and detached from the dishes. It is interesting that BDNF removal prompted an attempt to reenter S-phase, as judged by the increase in the number of BrdU-positive cells.

We finally wanted to analyze the state of phosphorylation of pRB, by means of a gel mobility shift (Fig. 4C). In untreated cells, the most abundant pRB species was hyperphosphorylated, as expected from active cycling cells. After treatments with RA and RA-BDNF, pRB was found to be progressively hypophosphorylated, in agreement with the observed withdrawal of these cells from the cell cycle. Consistent with the attempt of cells to

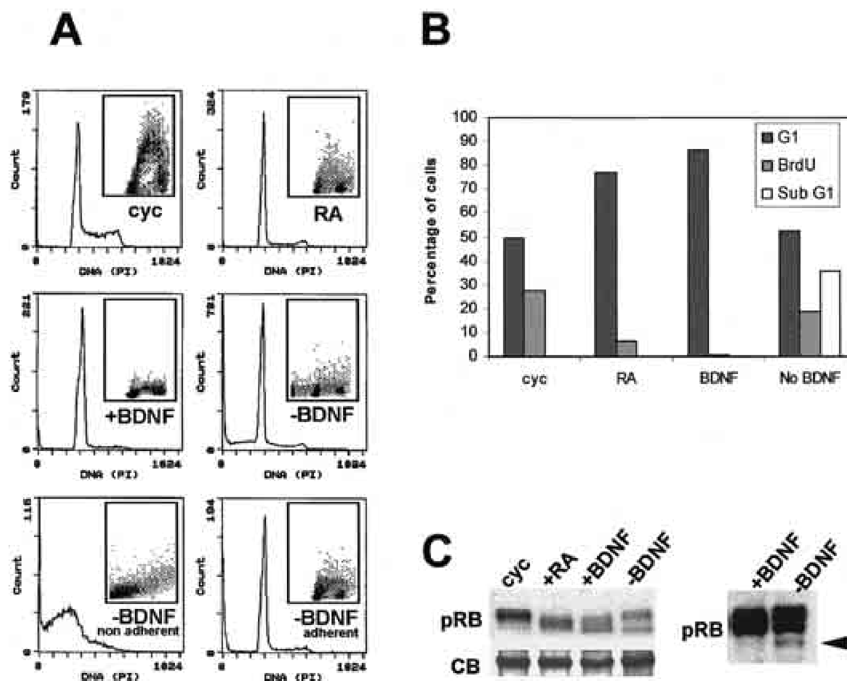

FIG. 4. RA-BDNF-treated cells are arrested in G1. Withdrawal of BDNF induces S-phase entry and apoptotic cell death. **A:** Flow cytometric analysis of cell cycle distribution [assessed by propidium iodide (PI) staining] during RA-BDNF differentiation. **Insets:** BrdU uptake of pulse-labeled cells under the same conditions. cyc, naive (cycling) cells; RA, 5 days of RA; +BDNF, 5 days of RA followed by 3 days of BDNF; and −BDNF, the same as +BDNF followed by 2 days in the absence of neurotrophin. **Bottom panels** show floating (nonadherent) and attached (adherent) cells from −BDNF condition. **B:** The proportion of cells in each cell cycle phase was quantified for the analyzed experimental conditions. **C, left panel:** Analysis of the state of phosphorylation of pRB, as deduced by gel mobility shift. The treatments are similar as in A, but the time of incubation with BDNF was 7 days. Equal loading of the lanes was verified by Coomassie Blue staining of the membranes, and the most abundant band is shown in the **lower panel** (CB). **Right panel:** Removal of BDNF also caused the appearance of a lower-molecular-weight pRB species (arrowhead), which is likely to be a caspase-cleaved fragment of this protein. Note that the gels in the left and right panels differ in the running gel conditions (see Materials and Methods).

reenter S-phase after BDNF removal, pRB showed a tendency to hyperphosphorylate, accompanied by the appearance of a novel, lower-molecular-weight pRB species, which is likely to be a caspase-cleaved fragment of this protein (Fig. 4C).

RA-BDNF-treated cells express several neuronal markers

The morphology of RA-BDNF-treated cells examined by phase-contrast microscopy showed that these differentiated cells resembled primary neurons. To analyze whether this phenomenon was accompanied by the expression of neuron-specific markers, we checked for the presence of several proteins by either immunocytofluorescence or western blot throughout the period of differentiation. Neurofilament triplet expression changed during the acquisition of fully neuronal phenotype. NF-L was predominantly expressed in untreated or RA-treated cells, whereas its expression decreased in cells treated with BDNF for 7 days as assessed by western blot (Fig. 5). NF-M was expressed in naive cells, and its expression considerably increased after 5 days of RA. After 7 days with BDNF, however, we could not detect this protein by western blot (Fig. 5).

Immunocytofluorescence revealed that NF-H was present in naive cells, with its localization being restricted to nuclear or perinuclear zones (Fig. 6). The same pattern of expression plus some labeling at the neurite extensions was observed in RA-treated cells, whereas cells treated for 7 additional days with BDNF showed a strong labeling at the neuritic processes, and besides perinuclear zones, cell bodies were also stained (Fig. 6). In BDNF-treated cells, MAP2 immunoreactivity was localized in the cytoplasm, with strong nuclear exclusion, and in short processes emanating from the cell body (Fig. 6). In naive and RA-treated cells, the pattern of distribution was similar except for the neuritic processes, with the majority of them being negative for this protein. In contrast, in RA-BDNF-treated cells, tau immunoreactivity was restricted to long, branched processes and was nearly undetectable in cell soma, whereas

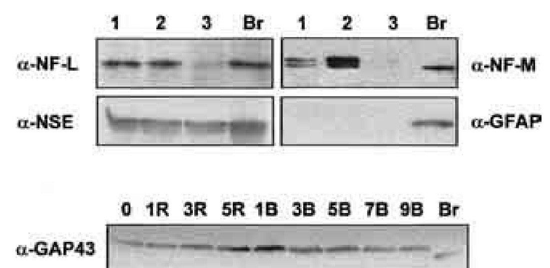

FIG. 5. RA-BDNF-differentiated SH-SY5Y express neuronal markers as shown by western blot analysis of the levels of NF-L, NF-M, NSE, GFAP, and GAP-43 in RA-BDNF-differentiated cells. **Top panel:** Cells were left untreated (lanes 1) or were treated with RA for 5 days (lanes 2) or with RA for 5 days followed by BDNF for 7 days (lanes 3). Note that cells were positive for NSE and negative for GFAP in all experimental conditions. **Bottom panel:** Cells were treated for the indicated days with RA (lanes designated R) or for 5 days with RA followed by the indicated days with BDNF (lanes designated B). Lane Br, mouse brain homogenates used as positive control.

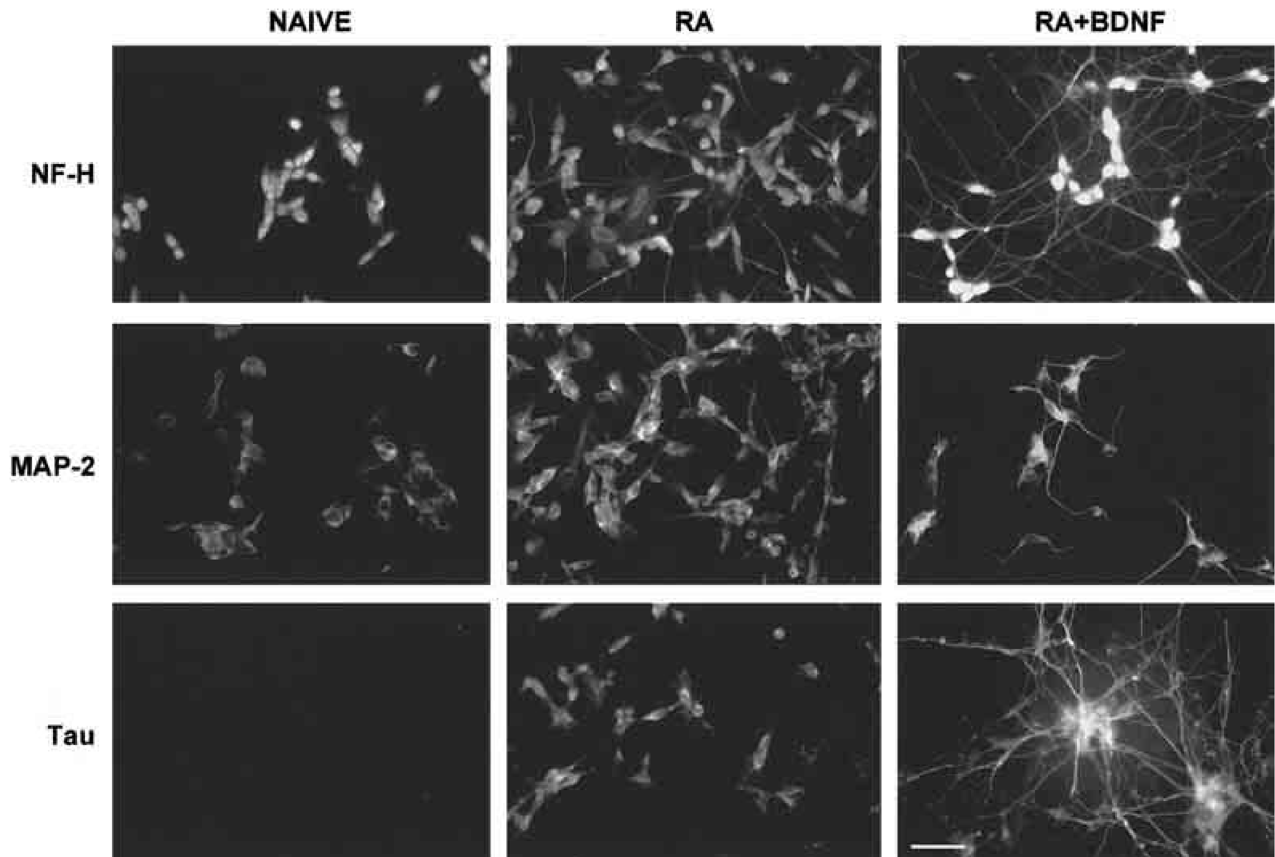

FIG. 6. RA-BDNF-differentiated SH-SY5Y express neuronal markers as shown by immunocytofluorescence for NF-H, MAP2, and tau of SH-SY5Y cells under different culture conditions: naive, 5 days of RA, or 5 days of RA followed by 7 days of BDNF. The expression of all markers increased along with the RA and BDNF treatment. NF-H arose from perinuclear areas (naive) and progressively extended to the rest of the cell body and neurites (5 days of RA and 7 days of BDNF), whereas MAP2 and tau were excluded from the nuclei, and at 7 days of BDNF they showed a complementary pattern of staining at the neurites with MAP2 staining the proximal portions of neurites (respect to the cell body) and tau the distal ones. Bar = 50 μm.

it was undetectable in naive cells. RA-treated cells showed a weak cytoplasmatic labeling that did not include neuritic processes. The pattern of labeling found in RA-BDNF-treated cells resembled the one found in cultures of primary neurons, in which MAP2 preferentially labels the somatodendritic domain whereas tau labels the axonal domain of neurons (Goedert et al., 1991). This observation suggests that RA-BDNF-differentiated cells are polarized.

The expression of NSE and GFAP was analyzed by western blot to confirm the neuronal lineage of these cells. As expected, NSE was present in all experimental conditions, whereas GFAP was never detectable (Fig. 5). Finally, analysis of the expression of GAP-43 revealed that this protein was transiently accumulated at day 5 of RA treatment and especially after the first day of BDNF exposure. These high levels returned to control values during the subsequent days of BDNF treatment (Fig. 5). This temporal pattern of expression coincides with the bulk of neurite outgrowth, in agreement with the proposed role of this protein in neurite elongation.

RA-BDNF-treated cells exhibit carbachol-evoked noradrenaline release

TPA-differentiated and naive SH-SY5Y cells have been reported to exhibit carbachol-evoked noradrenaline release (Scott et al., 1986; Murphy et al., 1991). To begin to characterize the neurotransmitter phenotype of RA-BDNF-treated cells, we performed noradrenaline release assays in untreated, RA-treated, or RA-BDNF-treated cells. A robust noradrenaline release was observed in each of the three conditions after carbachol stimulation, being 20.8 ± 0.88% in naive cells (values indicate the mean ± SEM percentage of noradrenaline released with respect to the total noradrenaline loaded), 17.2 ± 1.16% in RA-treated cells, and 8.9 ± 0.7% in RA-BDNF-differentiated cells. On the other hand, RA treatment has been shown to induce a slightly higher choline acetyltransferase activity in these cells, blocking TPA-induced noradrenaline production (reviewed by Pahlman et al., 1995). However, we failed to detect a significant choline acetyltransferase activity in any of the three experimental treatments (data not shown).

Finally, we measured the total content of several amino acid transmitters through the differentiation protocol by means of quantitative HPLC (Calvo et al., 1995). We were unable to detect significant changes in the total content of GABA, glycine, or taurine. However, glutamate levels decreased after 5 days of RA treatment (168.65 ± 24.6 ng in naive cells vs. 63.6 ± 16.3 ng in RA-treated cells) and were restored after RA-BDNF treatment (117.9 ± 13.9 ng). It should be noted that glutamate was by far the most abundant amino acid neurotransmitter present in any of the experimental conditions.

DISCUSSION

In this report we present a protocol of differentiation based on the sequential exposure of SH-SY5Y cells to RA and BDNF. This protocol yields homogeneous populations of fully neuronal differentiated cells. These cells are withdrawn from the cell cycle, express many of the typical neuronal markers, exhibit carbachol-evoked noradrenaline release, and are dependent on neurotrophic support for survival and differentiation. These characteristics make the cells obtained with this protocol very similar to primary neurons.

The effects of RA in SH-SY5Y are well documented. These include an attenuation of proliferation rate, extension of neuritic processes, and development of a slightly enhanced choline acetyltransferase activity (for review, see Pahlman et al., 1995). However, these studies generally do not consist of long-term analysis of the phenotype induced by RA treatment. In our hands, extended intervals in the presence of this agent favored the appearance of S-type cells, making further studies difficult to perform. This observation has been reported also by other authors in SH-SY5Y (Jensen, 1987; Hill and Robertson, 1997; Arcangeli et al., 1999) and other (Matsushima and Bogenmann, 1992) neuroblastoma cell lines. The phenomenon of transdifferentiation between N- and S-types seems to be common to most neuroblastoma cell lines, including those considered neuroblastic, like SH-SY5Y (Ross et al., 1983). This phenomenon may reflect the multipotential nature of neural crest cells from which neuroblastoma are presumed to arise. Therefore, it is considered that the prevalence of a given phenotype in a continuous neuroblastoma cell line is a consequence of slower rates of interconversion rather than loss of the potential to generate the other phenotype (Sadee et al., 1987). Moreover, in the case of neuroblastic subclones, during the processes of splitting and harvesting, S-type cells are selected against, owing to their tendency to remain attached to the culture plate (Jensen, 1987). Thus, the emergence of S-type cells after long-term treatments with RA could be primarily attributed to the spontaneous tendency of these cells to transdifferentiate when they are cultured for extended times without splitting them. The fact that S-type cells seem to be resistant to the growth inhibitory effects of RA would then contribute to the rapid expansion of these cells observed when SH-SY5Y are cultured in the presence of this agent. The possibility that RA may directly exert a dual differentiating effect over the two observed phenotypes cannot be excluded because this agent has been shown to promote differentiation of both adrenergic cells and melanocytes in neural crest cultures (Dupin and Ledouarin, 1995). However, the fact that treatment of SH-SY5Y cells with agents that promote growth inhibition of N-type cells such as NGF results in the appearance of S-type cells (Jensen, 1987) indicates that at least part of the transdifferentiation phenomenon is not triggered directly by RA.

Removal of RA after 5 days of treatment, followed by addition of BDNF in serum-containing medium, induced a more neuronal phenotype but did not circumvent the appearance of S-type cells as time in culture increased. In contrast, if serum was removed at this step, only residual amounts of S-type cells were present. A possible explanation for this behavior is that S-type cells, which do not express neuron-specific markers (Sadee et al., 1987; Ciccarone et al., 1989), fail to induce TrkB in response to RA and therefore die in the absence of trophic support. In this regard, it has been described that SH-EP, an S-type subclone of SK-N-SH, cannot survive in the absence of serum (see Leventhal et al., 1995).

The effects of BDNF on survival and differentiation indicate that TrkB receptors induced by RA are biologically active. In a previous report, we have shown that some of the relevant kinases involved in the signal transduction pathways mediated by BDNF become activated after addition of this neurotrophin to RA-pretreated SH-SY5Y cells. Moreover, the selective blockade of these pathways reverses the neurite-promoting and survival effects triggered by BDNF (Encinas et al., 1999). Previous studies have shown that RA promotes NGF survival responsiveness in cultured chicken sympathetic neuroblasts (Rodriguez-Tebar and Rohrer, 1991) by up-regulating the levels of TrkA (VonHolst et al., 1995, 1997). In contrast, RA inhibited the developmental increase in level of TrkA mRNA and the decrease in level of TrkC mRNA in mouse sympathetic neuroblasts (Wyatt et al., 1999). A recent work indicates that hippocampus-derived stem cell clones up-regulate expression of neurotrophin receptors when exposed to RA. Moreover, the sequential addition of RA and BDNF or NT-3 to these cells led to a significant increase in the number of mature neurons generated (Takahashi et al., 1999). Finally, RA and neurotrophins have been found to promote the survival and differentiation of pluripotent neural crest cells to a neuronal lineage (Sieber-Blum, 1991; Henion and Weston, 1994). These and other observations suggest that RA may be involved in the maturation of neural crest cells along a neuronal fate and that collaborative effects between RA and neurotrophins may be needed for the acquisition of a fully developed neuronal phenotype.

For the times analyzed, the viability of cells seemed to be conditioned by the continuous presence of BDNF in the culture medium. The removal of the neurotrophin

triggered a cell death that has many of the features of apoptosis, including DNA fragmentation (detected by TUNEL and flow cytometry), chromatin condensation, and activation of caspases. This antiapoptotic role of BDNF has been demonstrated in several types of primary neurons (see, for example, Lewin and Barde, 1996). It should be noted that the rate of cell death followed by BDNF withdrawal was strongly dependent on the cellular density, being slower in high-density cultures (data not shown). This could be explained in terms of an autocrine survival loop of insulin-like growth factor-II operating in SH-SY5Y cells switched to serum-free medium (Martin and Feldman, 1993). We have found that the optimal densities that yielded healthy cultures in the presence of BDNF and a relatively rapid cell death after starvation were $\sim 10^4$ cells/cm^2 (data not shown), which are sixfold lower than the threshold density required for autocrine insulin-like growth factor-II-mediated growth (Martin and Feldman, 1993).

Another consistent feature of cells generated by sequential exposure to RA and BDNF is its progressive withdrawal from the cell cycle. It is generally accepted that terminal differentiation of neuroblasts occurs once they have been arrested in G0 (reviewed by Ross, 1996). It is interesting that removal of BDNF prompted an attempt to reenter S-phase, as judged by BrdU uptake and hyperphosphorylation of pRB. Unscheduled S-phase entry has been associated with apoptotic cell death in several systems and is thought to be a consequence of deregulated E2F-1 release from pRB/E2F complexes (reviewed by Kasten and Giordano, 1998; Macleod, 1999). In RB-null mice, aberrant S-phase entry and concomitant apoptotic cell death are observed in differentiating cells, which should be exiting the cell cycle to become postmitotic (Lee et al., 1994; Morgenbesser et al., 1994). Moreover, ectopic expression of viral genes that interferes with E2F-1 binding to pRB also results in apoptotic cell death (Howes et al., 1994; Pan and Griep, 1994, 1995; Symonds et al., 1994). It is interesting that the massive apoptotic cell death observed in RB$-/-$ mice is abrogated by targeted disruption of E2F-1 (Tsai et al., 1998). Conversely, overexpression of E2F-1 causes apoptosis in mouse fibroblast cell lines after the cells enter S-phase (Qin et al., 1994; Shan and Lee, 1994; Wu and Levine, 1994).

In conclusion, the model presented herein accomplishes many of the characteristics presented by primary cultures of neurons. In particular, its trophic dependence toward BDNF makes this model system a suitable tool to approach the phenomenon of PCD and, more specifically, the relationship between this phenomenon and the cell cycle. Moreover, this model offers a convenient system to explore the therapeutic potential of neurotrophins in neurodegenerative diseases. Some differentiation procedures exist that yield fully mature human neuron-like cells. However, their trophic dependencies are not well established as most of them require serum factors for survival. Furthermore, many of the above models are time-consuming and require the use of antimitotic drugs that have been shown to be toxic for some primary cultures of neurons (Dessi et al., 1995; Sanz-Rodriguez et al., 1997; Anderson et al., 1999). In our model system, the use of antimitotics can be avoided, although SH-SY5Y cells have the ability to give rise to many neural crest derivatives. That the sequential use of RA and BDNF commits the vast majority of these cells to a neuronal phenotype suggests that this treatment could be mimicking some of the developmental signals that commit pluripotential neural crest cells to a neuronal fate.

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