Molecular Neurobiology

ISSN0893-7648/95/10(2-3): 105-114/$6.00

Treatment of PC12 Cells by Nerve Growth Factor, Dexamethasone, and Forskolin

Effects on Cell Morphology and Expression of Neurotensin and Tyrosine Hydroxylase

Thierry Caillaud, Wei Yuan Xu-van Opstal, Valérie Scarceriaux, Claude Billardon, and William Rostene*

Institut National de la Santé et de la Recherche Médicale, Unité 339, Hôpital Saint Antoine, 184 rue du faubourg Saint Antoine, 75571 Paris cedex 12, France

Abstract

Several lines of anatomical, neurochemical, electrophysiological, and behavioral evidence suggest the existence of physiological interactions between neurotensin (NT) and the brain dopaminergic systems. Thus, NT has been shown to exert a neuroleptic-like action and could be implicated in the pathogenesis and treatment of schizophrenia. It is thus of particular importance to develop in vitro cell culture systems as models to study such interactions. Rat adrenal pheochromocytoma PC12 cells, which expressed high levels of tyrosine hydroxylase, were used in the present study. In contrast to rat brain cells in primary cultures, PC12 cells did not express functional NT receptors. However, they were able to express both NTmRNA and NT in response to NGF, forskolin, and dexamethasone. Those neurochemical modifications furthermore may be related to changes in the morphology of the PC12 cells in response to NGF, forskolin, and dexamethasone alone or in combination. These data suggest that PC12 cells may provide a useful model to study in vitro the regulation of both catecholamine and neurotensin phenotypes.

Index Entries: PC12 cells; tyrosine hydroxylase; neurotensin; neurotensin receptor; cell morphology; image analysis; radioimmunoassay; Northern blots; growth factors; glucocorticoids.

Introduction

Neurotensin (NT) is a tridecapeptide isolated from bovine hypothalamus (Carraway and Leeman, 1973), which exerts a wide range of biological actions both in the gastrointestinal tract and in the central nervous system (CNS) (Kitabgi et al., 1985; Kitabgi and Nemeroff, 1992). In the CNS, substantial evidence has been brought up indicating that NT

*Author to whom all correspondence and reprint requests should be addressed.

acts as a modulator of dopamine (DA) transmission, notably in the hypothalamus and midbrain (Gudelsky et al., 1989; Kasckow and Nemeroff, 1991; Rostène et al., 1992). Some in vivo studies had previously demonstrated that NT could stimulate the electrical activity of nigrostriatal, mesolimbic, and cortical dopaminergic neurons (Pinnock, 1985; Shi and Bunney, 1991) and facilitate DA release (Blaha et al., 1990). This is consistent with data obtained in primary cultures of rat mesencephalic cells where we found that functional NT receptors were located on cell bodies and neuritic processes of tyrosine hydroxylase (TH) expressing neurons (Brouard et al., 1992). The major difficulty in handling such primary cultures lies in their limited lifespan and their heterogeneity with a variety of cells at different stages of development. Moreover, paucity of the cells of interest dispersed among the whole population frequently hinders their detection. In contrast, permanent cell lines can be propagated in rather homogeneous populations. A number of tumor cell lines, such as the PC12 cell line, have been thus developed. This cell line was derived from a rat adrenal medullary tumor and can synthesize and secrete catecholamines, such as dopamine (DA) and norepinephrine (Greene and Tischler, 1976).

It was clearly shown that on treatment with nerve growth factor (NGF), PC12 cells acquire a neuronal sympathetic-like phenotype by promoting neurite outgrowth associated with a progressive arrest of cell division (Greene and Tischler, 1976; Damon et al., 1990) and induction of electrical excitability (Dichter et al., 1977). It was also reported that stimulation with NGF, the synthetic glucocorticoid dexamethasone (DEX), and the activator of adenylate cyclase, forskolin (FSK), increased NT content in PC12 cells (Tischler et al., 1986).

The gene encoding neurotensin/neuromedin N precursor has indeed been recently cloned and several *cis*-regulatory elements have been therein localized (Kislaukis et al., 1988; Kislaukis and Dobner, 1990) such as a consensus AP1 site, the binding sequence of the proto-oncogenes c-fos and c-jun, two cAMP response element sites (CRE), and a glucocorticoid response element site (GRE).

Therefore, given the particular sensitivity of these cells to several inducers (Tischler et al., 1986), we have tried in this work related to the effects of growth factors on cell differentiation to quantitatively estimate the effects of single or combined treatments with NGF, DEX, and FSK on the morphology of PC12 cells by means of image analysis processing and on the synthesis and expression of both TH and NT in these cells.

In addition, considering that permanent cell lines may not retain all the differentiated traits of a neuronal cell, we wanted to further characterize PC12 cells with respect to the results obtained in primary neuronal cell cultures (Brouard et al., 1992) and more particularly investigate the presence of putative NT receptors.

Methods

Cell Culture and Morphological Studies

PC12 cells were obtained from N. Faucon-Biguet (Cellular and Molecular Neurobiology Laboratory, CNRS Gif-sur-Yvette, France). They were grown routinely in 75 cm^2 flasks (Corning, Corning, NY) in RPMI 1640 supplemented with 10% horse serum (Boehringer Mannheim, Germany), 5% fetal calf serum (IBF), and 20 mg/mL gentamycin. Cultures were maintained in a water-saturated atmosphere with 5% CO_2 at 37°C. They were subcultivated every week from near confluent cultures with a 1:4–1:6 ratio. The adhering cells were mechanically detached from the culture flask by gentle flushing of culture medium. The medium was changed three times a week.

In all experiments, PCl2 cells were plated in basic culture medium or medium supplemented with either 100 ng/mL 2.5 S mouse salivary gland NGF (Sigma, St. Louis, MO), $10^{-6}M$ DEX (Sigma), $10^{-6}M$ FSK (Sigma), or the combination of the three agents.

For morphological studies, PC12 cells were plated on 6-well tissue culture dishes (Costar, France). Triplicate cultures were untreated or treated as mentioned earlier. The cells were fixed in 1% glutaraldehyde.

An automated cell culture analysis system, "Trakcell," we recently developed was used in the present studies (Xu-van Opstal et al., 1994). It was composed of a PC AT compatible (with a 80386 cpu, a 80387 mathcoprocessor, 4 MB of RAM), a CCD camera, a video digitizer board (1 MB of frame buffer RAM), an inverted Nikon Diaphot microscope equipped with a 20× long distance objective, Hoffman modulation contrast optics, and a motorized stage (Biocom, les Ulis, France).

Each digitized image represented a region of 403 × 287 nm. The nominal spatial resolution was thus 0.787 × 0.561 nm/pixel.

Several parameters were determined:

1. Shape factor was used as a measurement of the circularity of the cells; a shape factor of 1 corresponds to round cells; it is lower than 1 if the cell is not round.
2. Equivalent diameter can be defined as the diameter of an irregular shaped cell if this cell was round. It gives an approximation of the cell size and is defined as $2\,(\text{surface}/\pi)^{1/2}$.
3. Neuritic processes: A cellular extension was considered as a neuritic process when its width was inferior or equal to half of the equivalent diameter of the cell body. Such a parameter allows determination of the number of proximal processes, whatever the length of the neurite.

NT Production and NT Binding

The cells were plated on 12-well tissue culture dishes (Costar) and were treated as indicated earlier for 0–6 d. The cells were harvested by scraping from the culture dish in ice-cold HCl; the suspension was then boiled for 10 min and centrifuged at 3500*g* for 8 min. The supernatants were kept frozen until used. Aliquots were taken for protein content determination using the Bradford Coomassie Blue method (Bradford, 1976).

NT-like immunoreactivity was measured in cell extracts by radioimmunoassay using an antibody recognizing NT and NT 1-11 but neither NT 8-13 nor Neuromedin N. The antibody was used at a 1:15,000 dilution. The radiolabeled ligand [^{125}I]-Tyr^{3}NT was prepared as previously described (Sadoul et al., 1984). The radioimmunoassay was subsequently performed as previously described (Schimpff et al., 1992).

For NT binding, PC12 cells were plated on 24-well tissue culture dishes and treated as described earlier. At the end of the incubation period, the culture medium was removed, the cells were washed, and then incubated for 10–120 min with either, 50 m*M* Tris-HCl buffer, pH 7.4, or serum-free culture medium both containing $10^{-3}M$ 1,10 orthophenanthroline (Coger), 0.2 mg/mL bacitracin (Sigma), 0.2% BSA (IBF), and 0.4 n*M* [^{125}I]-Tyr^3 NT. The experiments were performed either at 4 or 37°C. The nonspecific binding was assessed by adding $10^{-6}M$ unlabeled NT to the incubation medium. The cells were then washed three times at 4°C with either serum-free medium or Tris-HCl buffer and lysed in 0.1*N* NaOH. The radioactivity was counted in a γ counter (Packard) (Brouard et al., 1992).

Northern Blot Analysis

Preparation of Total RNA

Treated cells with NGF + forskolin + dexamethasone for 6 d or untreated cells were plated on 100-mm tissue culture Petri dishes. The medium was removed and the cells were washed once in ice-cold phosphate buffered saline (PBS, Gibco, Gaithersburg, MD) and solubilized in the dish by 1 mL of RNAzol (Bioprobe). Total RNA was extracted as indicated by the manufacturer. Rat adrenal medulla (positive control for TH mRNA) were dissected from the adrenal gland of adult Wistar female rats and kept frozen at -80°C until used. Hippocampal tissue (positive control for NT mRNA) and cerebellum (negative control for NT mRNA) were dissected from brains of newborn and 6-d-old rats. Total RNA

was prepared by using 2 mL of RNAzol for 100 mg of adrenal medulla or brain tissue.

Electrophoresis of Total RNA

Total RNA obtained either from PC12 cells (5 μg) or adrenal medulla, hippocampus, and cerebellum tissues (20 μg) was applied to a 1% agarose, 6% formaldehyde gel and processed for electrophoresis. Subsequently, RNA was transferred overnight by capillarity to a nylon membrane (Hybond, Amersham, Arlington Heights, IL) in 20X SSC (1X SSC: 150 m*M* NaCl, 1.5 m*M* sodium citrate, pH 7) and crosslinked by heating at 80°C for 2 h.

Hybridization of TH mRNA

A tyrosine hydroxylase 45-mer oligonucleotide probe (complementary to the 1442–1487 nucleotides of rat cDNA) was radiolabeled using a terminal transferase kit (Amersham) in the presence of [α-^{32}P]dATP (3000 Ci/mmol, Amersham). The filters were prehybridized for at least 2 h in 50% formamide, 6X SSC, 5X Denhardt's solution (50% Denhardt's solution: 1% polyvinylpyrrolidone, 10% Ficoll, 0.02% bovine serum albumin), 0.5% SDS, and 100 mg/mL heat denaturated salmon sperm DNA.

The filters were hybridized for 18–24 h at 42°C in 50% formamide, 6X SSC, 5X Denhardt's solution, 0.5% SDS with 1 pmol of the labeled oligonucleotide probe.

The filters were washed twice at room temperature in 2X SSC, 1% SDS for 10 min, twice in the same mixture for 15 min, twice in 1X SSC, 0.1% SDS at 42°C for 15 min. Autoradiograms of the filters were obtained with X-Omat films (Kodak) exposed at –80°C overnight.

Hybridization of NT mRNA

prNT4 was constructed by ligating a 336 basepair (bp) *Eco*RV/*Bgl*II fragment (nucleotides 626–961) of the rat NT/N gene into *Bam*H1/*Sma*1 digested pGEM4 (generous gift of P. Dobner, University of Worcester, Worcester, MA). A 221-bp fragment *Hind*/*Eco*R1 from prNT4 (containing 201 nucleotides of NT cDNA) was inserted into the polylinker cloning sites of M13 mp18 vector (Boehringer). The resulting construct was used as a template for preparing a uniformly labeled probe by annealing a 15-base M13 universal primer followed by primer extension using DNA polymerase large fragment [α-^{32}P]dCTP (3000 Ci/mmol) and unlabeled deoxynucleotides (Williams et al., 1986).

The primer extension was followed by a *Eco*R1 restriction digestion. The probe was purified using a 5% polyacrylamide denaturing gel electrophoresis containing 8*M* urea. The fragments were visualized by radioautography and electroeluted from the gel as described (Williams et al., 1986). Prehybridization was performed as described for TH mRNA. The filters were subsequently hybridized with a solution containing 10^6 cpm/mL of the NT probe for 18–24 h. The filters were washed twice at room temperature in 2X SSC, 0.5% SDS for 10 min, and finally twice in the same mixture at 42°C for 15 min. Autoradiograms of the filters were obtained by exposition with X-Omat films (Kodak) at –80°C for 5 d.

Statistical Data

The statistical analysis (mean ± SEM) was performed either by Student's *t*-test or by Dunnett's test.

Results

In order to see whether several factors, among them growth factors, may influence PC12 cell differentiation, and if such effect may be related to changes in endogenous NT, treatments of the PC12 cells with FSK, DEX, and NGF were investigated on both cell morphology and neurochemical modifications as described in the following.

The morphological data are illustrated in Fig. 1 and the quantitative results presented in Fig. 2. Control cells were mostly polygonal, a few of them being spherical with no cellular processes.

Cultures grown in the presence of DEX alone showed no significant changes of the cell shape but a twofold increase in the cell size

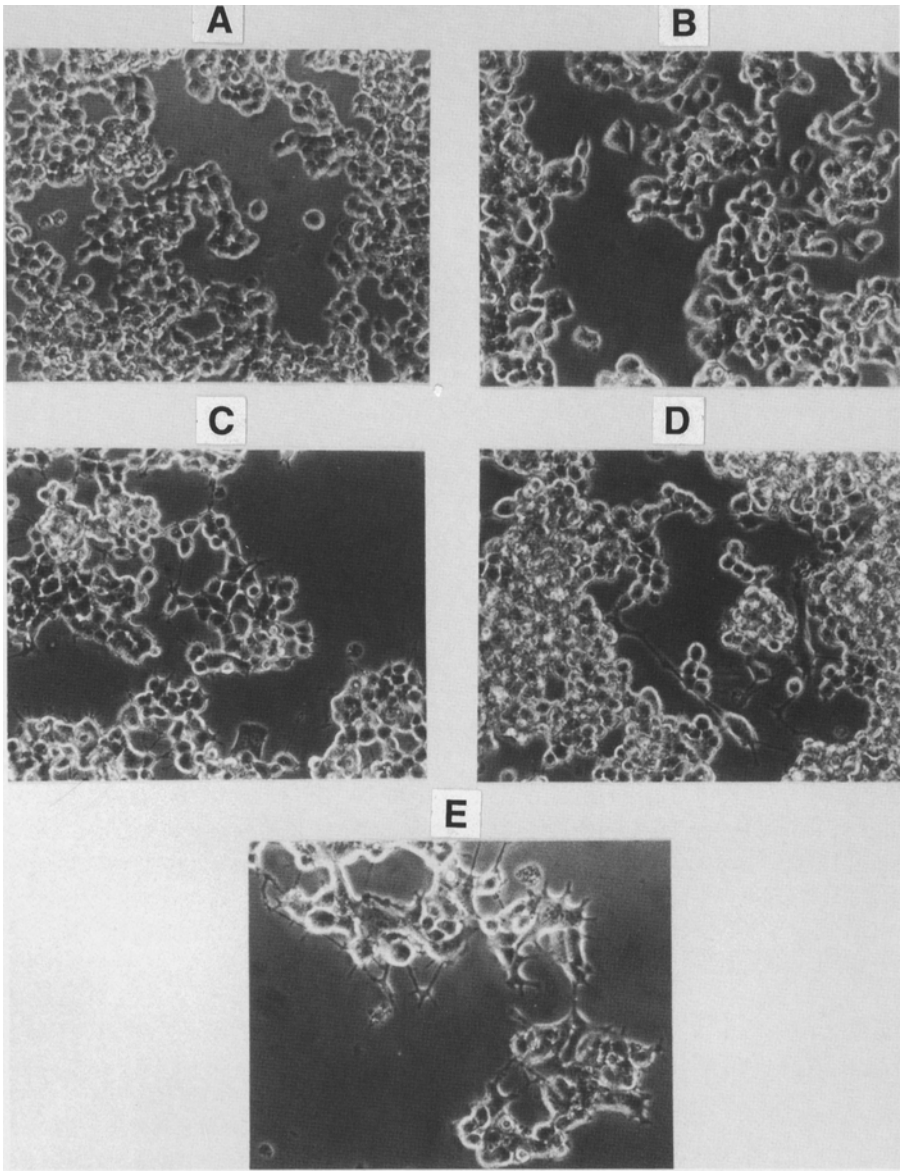

Fig. 1. PC12 cell morphology after 6 d of treatment **(A)** Control. **(B)** Dexamethasone (1 μ*M*). **(C)** Forskolin (1 μ*M*). **(D)** NGF (100 μg/mL). **(E)** NFD NGF + FSK + DEX.

(16.15 ± 0.71 μm vs 8.17 ± 0.38 μm for controls). Neither control nor treated cells exhibited any neuritic processes.

Treatment of the cells with FSK generated neuritic process outgrowth in most of the cells but with a small number of neurites per cell (approx 2/cell). This tranformation of the cell shape resulted in a marked drop of the shape factor (0.17 ± 0.01 vs 0.82 ± 0.01 in controls). This was correlated to a twofold increase of the cell size as it was found when the cells were treated with DEX.

Treatment with NGF alone gave similar results to those observed with FSK but with a better efficiency (four neurites vs two neurites in FSK-treated cells). Neuritic processes

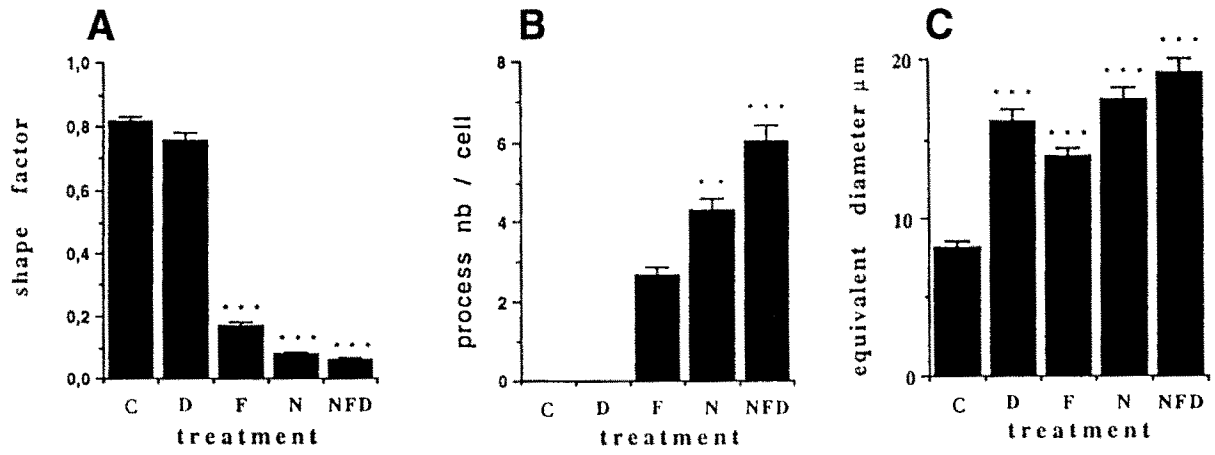

Fig. 2. Effect of DEX, FSK, NGF, and combined treatment on PC12 cell morphology. C: Control; D: Dexamethasone (1 µ*M*); F: Forskolin (1 µ*M*); N: NGF (100 ng/mL); NFD: NGF + FSK + DEX. **(A)** Shape factor. **(B)** Number of neuritic processes per cell. **(C)** Equivalent diameter of cell bodies. Statistical differences vs control (A,C) or forskolin (B) were assessed using Dunnett's test (***$p < 0.001$; **$p < 0.01$).

appeared to be longer and thicker when treated with NGF alone than with any other treatment.

The most important morphological differentiation was obtained with the combination of the three drugs. Each cell yielded about six processes. Moreover, a two- to threefold increase in the size of the cell bodies was observed (19.16 ± 0.8 µm vs 8.17 ± 0.38 µm in controls) as illustrated by the low values of the shape factor (0.06 ± 0.01 vs 0.82 ± 0.01 in controls).

Effect of NGF, DEX, and FSK on NT Content of PC12 Cells

NT-like immunoreactivity was measured following the different drug treatments that were applied over a 6-d period of time. As shown in Fig. 3, NT levels slightly increased during the 6-d incubation of the PC12 cells.

FSK (1 µ*M*) induced a constant increase in NT content of the cells. This effect was persistent whatever the length of treatment and reached a fourfold increase after 6 d.

DEX (1 µ*M*) slightly increased NT content of the cells during the first 4 d of treatment. After 6 d, NT content of the cells reached similar levels to those observed with FSK.

In contrast to what was observed with FSK and DEX, NGF (100 ng/mL) seemed to act primarily during the first 2 d of treatment. The combination of the three agents was necessary to induce the maximal level of NT content. It is worth noting that, although the kinetics of action of each drug taken separately was different, the combined treatment induced the same pattern of response when compared with the FSK alone, but exhibiting a sixfold increase in NT levels after a 6-d treatment.

NT Binding

A number of experimental conditions were tested as described in the Methods section. The cells were either unstimulated or treated with the combination of the three drugs. Similar data were obtained whatever the incubation temperature or the incubation medium used, and no specific binding of [^{125}I]-NT could be detected either in control or in treated cells.

TH and NT mRNA Production

mRNA production was demonstrated using Northern blot analysis on control cells and cells treated with the combination of FSK, DEX, and NGF (Fig. 4).

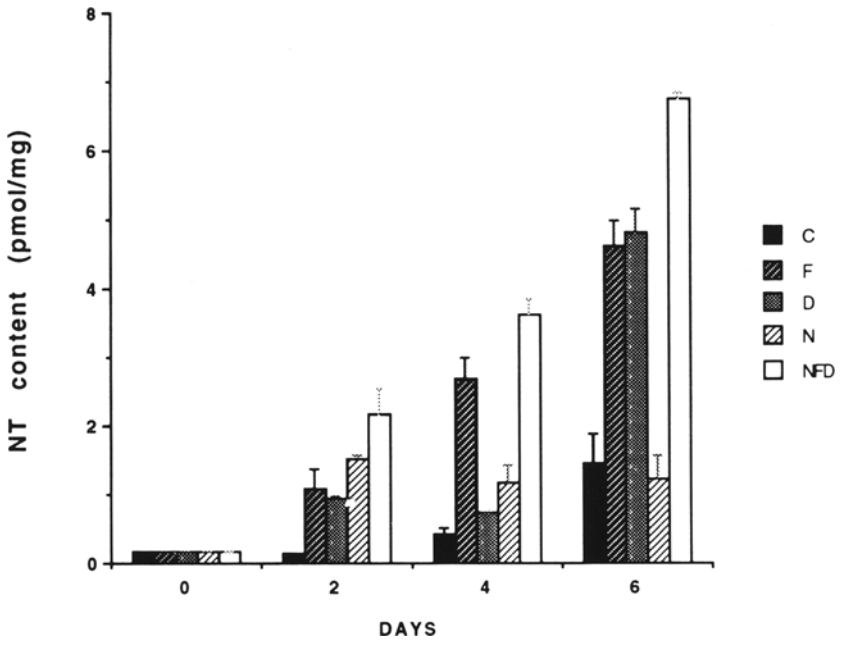

Fig. 3. Effect of DEX, FSK, NGF, and combined treatment on PC12 cell NT content. C: Control; D: Dexamethasone (1 μ*M*); F: Forskolin (1 μ*M*); N: NGF (100 ng/mL); NFD: NGF + FSK + DEX. The values are expressed in pmol of NT per mg of protein. Each value is the mean ± SEM of three different cultures.

TH mRNA

Hybridization of PC12 cell RNA with a TH 45-mer oligonucleotide probe revealed a mRNA major species at about 1.8–1.9 kb corresponding to TH mRNA and a minor species at about 4–5 kb corresponding most probably to a TH pre-mRNA (Saadat et al., 1987; Icard-Liepkalns et al., 1992).

A sixfold increase in TH mRNA expression was observed in the treated cells when compared to the control cells. Moreover, a third band of around 8.6 kb was detected in NFD-treated cells (Fig. 4B).

NT mRNA

Hybridization of PC12 cell RNA with the M13 NT probe yielded a single 1-kb band of NT mRNA that can be detected in the treated cells exclusively, whereas no NT expression was found in control cells in our experimental conditions (Fig. 4A). This 1-kb transcript has already been described by Dobner et al. (1992) who demonstrated that 1-kb mRNA greatly predominated in gastrointestinal tract cells. In contrast, it is worth noting that two mRNA species (approx 1 and 1.5 kb) were detected in the brain positive controls (hippocampus).

Discussion

PC12 cells originated from a rat adrenal pheochromocytoma were shown to synthesize catecholamines such as norepinephrine and dopamine (Greene and Tischler, 1976). They can be differentiated into a sympathetic neuronal phenotype when treated with NGF (Greene and Tischler, 1976; Damon et al., 1990). It was shown that NGF was able to promote neurite outgrowth associated with a progressive stop of cell division (Greene and Tischler, 1976). In the present work, we demonstrated that a combined treatment of PC12 cells with NGF, glucocorticoids, and an activator of adenylate cyclase, forskolin, increased both tyrosine hydroxylase and neurotensin expression.

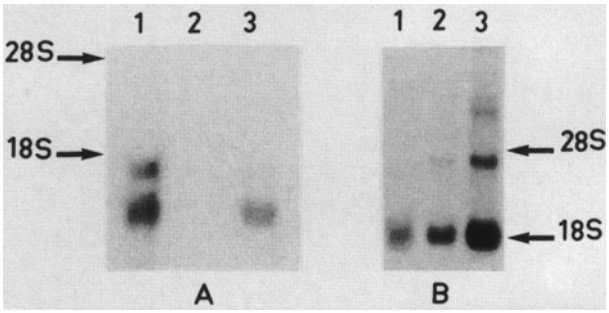

Fig. 4. Northern blots of NTmRNA and THmRNA **(A)** NTmRNA 1: Rat hippocampus. 2: Control PC12 cells. 3: NFD (NGF + FSK + DEX) PC12-treated cells. **(B)** THmRNA 1: Rat adrenal medulla. 2: Control PC12 cells. 3: NFD (NGF + FSK + DEX) PC12-treated cells.

Moreover, our data confirm those of Tischler et al. (1986) obtained in another subline of PC12 cells showing that the same drugs were able to enhance endogenous NT levels. These findings are of interest with regard to the close interactions occurring between neurotensin and dopamine in the CNS. Hökfelt et al. (1984) reported that some DA containing cells in the rat midbrain were able to coexpress NT. A neuromodulatory role of this peptide on central DA systems has been extensively discussed in books and reviews (Kitabgi and Nemeroff, 1992; Rostène et al., 1992). In connection with these data, we recently described that more than 80% of the TH-expressing neurons presented functional NT receptors in primary cultures of rat mesencephalic cells (Brouard et al., 1992). These receptors were located both on cell bodies and neuritic processes (Brouard et al., 1992). In the present work, we thus checked whether such NT receptors may be similarly found on differentiated PC12 cells, since NT binding sites have been already detected in normal rat medullary adrenal cells (Goedert et al., 1984). In contrast to what was observed in mesencephalic and medullary adrenal cells, no specific NT binding was found, whatever the drug treatment used.

A combined treatment of the cells with FSK, DEX, and NGF dramatically increased TH mRNA production. It is in agreement with previous works by others that used those different inducers independently (Baetge et al., 1981; Lewis et al., 1983). Similarly, the combined treatment enhanced NT mRNA expression that may be linked to the increase in NT content observed in these cells with a drug-dependent time course. In contrast to FSK, which produced a constant increase in NT levels, and to DEX, which was mainly active after a 3-d treatment, NGF rapidly enhanced the cell NT content within the first 2 d. The combination of the three drugs yielded the same pattern of response as did FSK alone, but with a higher amplitude. These findings may support the assumption that the different time courses are probably related to various transduction mechanisms involved in the effects of the different drugs. Indeed, in PC12 cells, as well as in the central and peripheral nervous system, NGF was shown to exert its effects through the activation of the signaling capacity of *trk A*, a receptor tyrosine kinase that, on interaction with NGF, becomes phosphorylated on tyrosines and thereby acquires the potential to interact with intracellular signal transducing proteins (Obermeier et al., 1994). Pretreatment of PC12 cells with selective inhibitors recently suggested that NGF requires tyrosine kinase, phospholipases C and A2, and calcium release from intracellular stores for the early stage of action of NGF to induce neurite outgrowth (Tsukada et al., 1994). It becomes quite clear from recent data that the neuronal differentiation induced by NGF also involved several different early gene induction as well as redundant signal transduction pathways (Stephens et al., 1994). This action of NGF through intracellular transduction pathways may be related to the more rapid effect of NGF on NT content than the one observed with FSK or DEX. Concerning the latter, it probably involved interactions with nuclear proteins, which requires a long-lasting effect (McEwen et al., 1986).

The action of FSK and DEX on NT content and NTmRNA is consistent with the presence of the necessary *cis*-regulatory elements CRE and GRE previously described on the NT gene

(Kislaukis et al., 1988; Kislaukis and Dobner, 1990). The synergy between the effect of the two drugs is in agreement with the functional interdependence of the different response elements on the NT promoter (Kislaukis and Dobner, 1990). It has been proposed that such synergy between glucocorticoids and cAMP could be owing to an enhanced DNA binding of the glucocorticoid receptor to GRE following protein kinase A activation, through phosphorylation of the receptor itself, or phosphorylation of associated proteins (Nordeen et al., 1994).

Additional information was brought up in the present work by the investigation on the developmental differentiation patterns, evidenced by the morphological changes that were studied by means of quantitative image analysis. The presence of neuritic processes started after 2 d of treatment with NGF. These processes were longer and thicker than those obtained with any other treatment.

The NGF-elicited neurite outgrowth was reported to be independent of the cAMP pathway (Richter-Landsberg and Jastorff, 1986; Damon et al., 1990), although the neurite formation can be observed following FSK treatment alone. As seen for the induction of NT, multiple second messenger systems can operate singularly or concomitantly to mediate a variety of growth factor actions (Damon et al., 1990). The combined treatment with FSK, DEX, and NGF induced an additive effect on the number of processes per cell as compared with each drug treatment, but we observed that neurites were thinner than those found in NGF-treated cells. This could be linked to an interaction between the three drugs, such as a DEX-induced reduction of the NGF receptor expression (Foreman et al., 1992).

The maturation process induced by the three different drugs leads to a neuronal phenotype in PC12 cells. These morphological parameters may be related to endogenous neurochemical modifications, as shown in the present work, for both the catecholaminergic and peptidergic phenotypes. It has been previously reported that the maximal increase of TH occurred when the complete cellular development of neuronal cells was achieved in cultures of rat brain cells (Porter et al., 1990). As is the case for several neurotransmitters, the increase in NT production could be linked to the existence of neuritic processes. Indeed, it does not seem to be the case, since increase in NT content can be observed in DEX-treated cells in which no processes have been observed. This observation may be related to a previous work of Tischler et al. (1983) showing that cells maintained in spinner cultures, under which conditions they do not form any processes, can still produce NT. It suggests that the peptide may be stored in the cell cytoplasm until it can be transported in the mature neuronal processes as was clearly demonstrated for NT both in the central and peripheral nervous system (Kessler and Beaudet, 1989; Castel et al., 1990).

In conclusion, although PC12 cells lacking functional NT receptors under our experimental conditions cannot be directly used as substitutes for neuronal primary cultures (Brouard et al., 1992, 1993; Scarcériaux et al., 1994), they may provide a useful tool for the in vitro study of the regulation by growth factors and other inducers of both DA and NT systems.

Acknowledgments

We would like to thank N. Faucon-Biguet (Cellular and Molecular Neurobiology Laboratory, CNRS, Gif-sur-Yvette, France) who provided us with the PC12 cells and who helped us with the THmRNA determination. Supported by the MESR, France.

References

Baetge E. E., Kaplan B. B., Reis D. J., and Joh T. H. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 1269.

Blaha C. D., Coury A., Fibiger H. C., and Phillips A. G. (1990) *Neuroscience* **34,** 699.

Bradford M. M. (1976) *Anal. Biochem.* **72,** 248.

Brouard A., Pélaprat D., Boja J. W., Carroll F. I., Vial M., Kuhar M. J., et al. (1993) *Dev. Brain Res.* **75,** 13.

Brouard A., Pélaprat D., Dana C., Vial M., Lhiaubet A.-M., and Rostène W. (1992) *J. Neurosci.* **12,** 1409.

Carraway R. E. and Leeman S. E. (1973) *J. Biol. Chem.* **248,** 6854.
Castel M. N., Malgouris C., Blanchard J. C., and Laduron P. M. (1990) *Neuroscience* **36,** 425.
Damon D. H., D'Amore P. A., and Wagner J. A. (1990) *J. Cell Biol.* **110,** 1333.
Dichter M. A., Tischler A. S., and Greene L. A. (1977) *Nature* **268,** 501.
Dobner P., Kislaukis E., and Bullock B. P. (1992) *Ann. NY Acad. Sci.* **668,** 17.
Foreman P. J., Taglialatela G., Jackson G. R., and Perez-Polo J. R. (1992) *J. Neurosci. Res.* **31,** 52.
Goedert M., Mantyh P. W., Hunt S. P., and Emson P. C. (1984) *Brain Res.* **299,** 389.
Greene L. A. and Tischler A. S. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2424.
Gudelsky G. A., Berry S. A., and Meltzer H. Y. (1989) *Neuroendocrinology* **49,** 604.
Hökfelt T., Everitt B. J., Theordorson-Norheim E., and Goldstein M. (1984) *J. Comp. Neurol.* **222,** 543.
Icard-Liepkalns C., Faucon Biguet N., Vyas S., Robert J. J., Sassone-Corsi P., and Mallet J. (1992) *J. Neurosci. Res.* **32,** 290.
Kaschow J. and Nemeroff C. B. (1991) *Regul. Pept.* **36,** 153.
Kessler J. P. and Beaudet A. (1989) *J. Neurosci.* **9,** 466.
Kislaukis E. and Dobner P. R. (1990) *Neuron* **4,** 783.
Kislaukis E., Bullock B., McNeil S., and Dobner P. R. (1988) *J. Biol. Chem.* **263,** 4963.
Kitabgi P. and Nemeroff C. B. (1992) *Ann. NY Acad. Sci.* **668,** 1–374.
Kitabgi P., Checler F., Mazella J., and Vincent J. P. (1985) *Rev. Clin. Basic Pharmacol.* **5,** 397.
Lewis E. J., Tank A. W., Weiner N., and Chikaraishi, D. M. (1983) *J. Biol. Chem.* **258,** 14,632.
McEwen B. S., De Kloet E. R., and Rostène W. H. (1986) *Physiol. Rev.* **66,** 1121.
Nordeen S. K., Moyer M. L., and Bona B. J. (1994) *Endocrinology* **134,** 1723.
Obermeier A., Bradshaw R. A., Seedorf K., Choidas A., Schlessinger J., and Ullrich A. (1994) *EMBO J.* **13,** 1585.
Pinnock R. D. (1985) *Brain Res.* **338,** 151.
Porter J. C., Kedzierski W., Aquila-Mansilla, N., and Jorquera B. A. (1990) *Endocrinology* **126,** 2474.
Richter-Landsberg C. and Jastorff B. (1986) *J. Cell. Biol.* **102,** 821.
Rostène W., Brouard A., Dana C., Masuo Y., Agid F., Vial M., et al. (1992) *Ann. NY Acad. Sci.* **668,** 217.
Saadat S., Stehle A. D., Lamouroux A., Mallet J., and Thoenen H. (1987) *J. Biol. Chem.* **262,** 13,007.
Sadoul J. L., Mazella J., Amar S., Kitabgi P., and Vincent J. P. (1984) *Biochem. Biophys. Res. Commun.* **120,** 812.
Scarcériaux V., Pélaprat D., Lhiaubet A. M., Schimpff R. M., Tramu G., and Rostène W. (1994) *Dev. Brain Res.* **81,** 128.
Schimpff R. M., Lhiaubet A. M., Vial M., and Rostène W. (1992) *Prog. Neuroendocrinimmunol.* **5,** 126.
Shi W. X. and Bunney B. S. (1991) *Brain Res.* **543,** 315.
Stephens R. M., Loeb D. M., Coperland T. D., Pawson T., Greelle L. A., and Kaplan D. R. (1994) *Neuron* **12,** 691.
Tischler A. S., Greene L. A., Kwan P. W., and Slayton W. W. (1983) *Cell Tissue Res.* **228,** 641.
Tischler A. S., Lee Y. C., Costopoulos D., Slayton V. W., Jason W. J., and Bloom S. R. (1986) *J. Neurosci.* **6,** 1719.
Tsukada Y., Chiba K., Yamazaki M., and Mohri T. (1994) *Biol. Pharm. Bull.* **17,** 370.
Williams D. L., Newman T. C., Shelness G. S., and Gordon D. A. (1986) *Methods Enzymol.* **128,** 671.
Xu-van Opstal W. Y., Ranger C., Lejeune O., Forgez P., Boudin H., Bisconte J. C., et al. (1994) *Microsc. Res. Tech.* **28,** 440.