Phenotypic Alterations of Neuropeptide Y, Vasoactive Intestinal Peptide and Choline Acetyltransferase in Rat Cultured Chromaffin Cells as Effected by Nerve Growth Factor and Glucocorticoid

Masafumi MORIMOTO1,2, Noriyuki MORITA1, Tomoyuki ICHIKAWA2 and Mitsuhiro KAWATA1

Department of Anatomy and Neurobiology1, Kyoto Prefectural University of Medicine, Kyoto; and Department of Anatomy and Embryology2, Tokyo Metropolitan Institute for Neurosciences, Fuchu, Tokyo, Japan

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Summary. We assessed changes in neuropeptide Y (NPY), vasoactive intestinal peptide (VIP) and choline acetyltransferase (ChAT) immunoreactivities when neonatal rat chromaffin cells were cultured in a medium containing nerve growth factor (NGF) or the synthetic glucocorticoid dexamethasone (DEX), examining whether their expression was correlated with the morphological changes induced by NGF and DEX. All of the chromaffin cells in culture were tyrosine hydroxylase (TH)-immunopositive regardless of whether they extended processes. TH-immunoreactive materials of NGF-treated chromaffin cells were distributed in all the cytoplasmic processes, even at the tips of growth cones. The percentage of NPY-positive chromaffin cells did not change markedly when treated with NGF or DEX throughout the 14 days in culture. The proportion of VIP-positive chromaffin cells increased gradually in the NGF-treated group and that of ChAT-positive cells in the group was similar to VIP. The morphological alterations induced by NGF were not correlated with the changes in proportions of NPY-, VIP- or ChAT-positive chromaffin cells. The percentages of VIP- or ChAT-immunoreactive chromaffin cells in the NGF-treated group showed much greater increases than those in the DEX-treated group. These findings suggest that NGF might modulate the phenotypic changes of neuropeptides and amines in rat chromaffin cells.

Adrenal chromaffin cells and sympathetic neurons arise from the same sympathoadrenal progenitors whose fate is determined by environmental signals (DOUPE et al., 1985a, b; ANDERSON and AXEL, 1986); glucocorticoids drive the progenitor toward a chromaffin fate (SEIDEL and UNSICKER, 1989; MICHELSON and ANDERSON, 1992), and nerve growth factor (NGF), ciliary neurotrophic factor (CNTF) and fibroblast growth factor (FGF) collaborate to drive the differentiation of the progenitors into sympathetic neurons (IP et al., 1994).

Although mature sympathetic neurons can not transform into chromaffin cells, chromaffin cells retain the capacity to transform into neurons (ALOE and LEVI-MONTALCINI, 1979) even when taken from mature animals (TISCHLER et al., 1993). In vitro, rat chromaffin cells are known to respond to NGF and differentiate into sympathetic neurons, while glucocorticoids inhibit the neuronal differentiation of chromaffin cells induced by NGF (UNSICKER et al., 1978; ANDERSON and AXEL, 1986). Our previous study of chromaffin cells showed that NGF induces an increase in the percentage of process-bearing cells, the number of total branches and the total length of processes, while DEX causes decreases in these markers of cytological differentiation of neurons (MORIMOTO et al., 1994).

Chromaffin cells apparently differ from sympathetic neurons in location and cytological features in vivo, but share common features of the same neuropeptides and catecholamines; subsets of postnatal rat sympathetic neurons and chromaffin cells contain neuropeptide Y (NPY) in addition to norepinephrine or epinephrine (LUNDBERG et al., 1982; DE QUIDT and EMSON, 1986; HENION and LANDIS, 1990). Small populations of postnatal sympathetic neurons and chromaffin cells contain vasoactive intestinal peptide (VIP), which is found in cholinergic neurons in adult animals (HÖKFELT et al., 1977; KONDO, 1985; TYRRELL and LANDIS, 1994).

These profiles of expression of neuropeptides and amines are dependent upon regulatory factors and
environmental cues (Zigmond et al., 1992; Hyatt-Sachs et al., 1993). In addition to these humoral factors, depolarization of preganglionic nerve fibers has been reported to affect the expression of neuropeptides and amines in the rat chromaffin cells (Doupe et al., 1985a; Henion and Landis, 1990).

There have been no reports of studies concerning the phenotypic alterations of neuropeptides in cultured chromaffin cells by NGF and glucocorticoid. It has been shown that most catecholaminergic neurons contain NPY and cholinergic neurons contain VIP in various neuronal systems (Elfrin et al., 1993). In the present study, we assessed the changes in NPY, VIP and choline acetyltransferase (ChAT) immunoreactivities when neonatal rat chromaffin cells were cultured in a medium containing NGF or DEX, and examined whether their expression was correlated with the morphological changes induced by these regulatory factors.

MATERIALS AND METHODS

Timed pregnant Sprague-Dawley rats were purchased from the Nippon Laboratory Animals Co. Ltd. (Osaka, Japan). Newborn rat pups were used within 24 h after birth. The rats were anesthetized by cooling on ice. Adrenal medullae from the newborn rats were dissected free of the cortex and cut into small pieces. Cells were washed in calcium/magnesium-free Hanks’ balanced salt solution (HBSS, GIBCO, USA) and incubated at 37°C for 60 min in calcium/magnesium-free phosphate-buffered saline (PBS) containing Collagenase/Dispase (1 mg/ml, Boehringer Mannheim, Germany). Chromaffin cells were purified by a modification of Henion’s method (Henion and Landis, 1992). Briefly, the cell suspension was then centrifuged at 150 x g for 3 min. Cells were resuspended in a minimum essential medium (MEM, GIBCO, USA) containing 10% fetal bovine serum (FBS, GIBCO) and dissociated by gentle trituration with a fire-polished glass pipette. The resulting cell suspension was carefully layered on top of density gradients consisting of 40% and 60% Percoll (Pharmacia, Sweden) in MEM, and centrifuged at 400 x g for 15 min at room temperature. After centrifugation, the 40% Percoll layer was collected and washed with a sixfold excess of HBSS. The density of the 40% Percoll layer was 1.052-1.074; most chromaffin cells were contained in this layer. The suspension was centrifuged at 150 x g for 5 min and the cells were washed with 2 ml of HBSS. The suspension was recentrifuged at 150 x g for 5 min. The cells were resuspended in 2 ml of MEM containing 10% FBS and incubated on non-coated tissue culture dishes. After 2 h incubation, the supernatant was collected and the number of viable cells was estimated by trypan blue exclusion. Approximately 500 cells were then plated on 10 mm glass coverslips coated with polyethyleneimine (Sigma, USA).

The basal growth medium for chromaffin cell cultures consisted of Liebovitz’s L15 (GIBCO)-CO₂ medium (Doupe et al., 1985a) supplemented with 10% FBS stripped of endogenous steroids by heat treatment and absorption with charcoal and dextran. The cultures were grown at 37°C in a 5% CO₂ environment for 2 days, 5 days, 7 days or 14 days in the following two experimental groups in media containing NGF (2.5, 20 ng/ml, GIBCO) or DEX (5 μM, Sankyo, Japan). Media were changed every 3 days. In experiments performed under depolarizing conditions, the cultures were grown with a serum-free L-15 CO₂ medium containing N-2 supplement (GIBCO), with potassium chloride (KCl; 40 mM) added to the medium.

Cultured cells were fixed with 4% paraformaldehyde in 0.1 M PBS plus 0.12 M sucrose for 30 min at room temperature, preblocked for 1 h at room temperature in a solution containing 0.1 M PBS, 8% normal goat serum and 0.3% Triton X-100, and then incubated with a mouse monoclonal antibody raised against tyrosine hydroxylase (TH; Incstar, USA, working dilution 1:2,000 diluted by PBS) and with a rabbit polyclonal antibody raised against NPY (Amersham, USA, working dilution 1:1,000) or VIP (Incstar, working dilution 1:1,000) or ChAT (Ichikawa et al., 1991, working dilution 1:500) overnight at 4°C. Cells were then incubated in FITC-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG (TAGO, USA, working dilution 1:200) for 2 h at 37°C. Controls lacking the primary antibody exhibited no specific staining. The secondary antibody against mouse IgG did not show any reactivity to the three rabbit polyclonal antibodies, and the secondary antibody against rabbit IgG did not show reactivity to the mouse monoclonal TH antibody. The percentage of the total population of chromaffin cells bearing processes was estimated by Nomarski modulation contrast microscopy.

RESULTS

Number of chromaffin cells

All of the chromaffin cells in culture were tyrosine hydroxylase (TH)-immunopositive regardless of whether they extended processes. TH-immunoreactive materials of NGF-treated chromaffin cells were
distributed in all the cytoplasmic processes, even at the tips of growth cones. There were no significant differences in the number of TH-immunopositive cells per coverslip between the NGF-treated and the DEX-treated groups throughout the culture period. In addition, the number of chromaffin cells did not significantly change from 2 days in vitro (2 DIV) to 14 DIV in both groups. In contrast, the number of chromaffin cells cultured without NGF or DEX was significantly decreased by 14 DIV compared with the NGF- or DEX-treated group. Alternatively, non-chromaffin cells were rarely observed on the coverslips of 2 DIV. Most of these were thought to be fibroblasts due to their morphology. The number of non-chromaffin cells slightly increased as the culture proceeded, but the portion of fibroblasts never exceeded 10% of the total cell number by 14 DIV.

**Percentage of process-bearing cells**

The percentage of process-bearing cells, which were thought to be differentiated into neurons, was progressively increased by the medium containing NGF (N/-) from 2 DIV to 14 DIV (Table 1). On the other hand, few process-bearing cells were observed in only DEX-treated (-/D) groups throughout the culture period.

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<th>2 DIV</th>
<th>5 DIV</th>
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<td>N/-</td>
<td>12.0±0.8</td>
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<td>-/D</td>
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**Neuropeptide Y (NPY)**

The percentage of NPY-immunoreactive cells among those immunopositive for TH did not show any marked changes during the culture period (Figs. 1, 2), and there were no significant differences between N/- and -/D groups from 2 DIV to 7 DIV (44.8±3.1% by N/- and 57.1±3.4% by -/D at 2 DIV, 45.2±4.4% by N/- and 42.9±3.2% by -/D at 5 DIV, 55.9±4.1% by N/- and 66.2±2.8% by -/D at 7 DIV). On 14 DIV, a significant difference was observed between the two groups; the data for N/- and -/D on 14 DIV were 32.8±5.3% and 61.2±3.5%, respectively (Mann-Whitney U test, p<0.05). In addition, there was a significant difference in the percentages of NPY-immunopositive cells of N/- group between on 7 DIV and 14 DIV; the data for 7 DIV and 14 DIV of N/- group were 55.9±4.1% and 32.8±5.3%, respectively (Mann-Whitney U test, p<0.05). Morphological changes induced by NGF were not correlated with the changes in the proportion of NPY-immunopositive chromaffin cells (Spearman rank correlation test, p=0.73).

**Vasoactive intestinal peptide (VIP)**

In the N/- group, the percentage of VIP-immunoreactive cells among those immunopositive for TH showed a slight increase during culturing (24.4±2.6% at 2 DIV, 31.9±3.2% at 5 DIV, 42.7±9.9% at 7 DIV and 45.4±8.8% at 14 DIV). In contrast, that of VIP-immunopositive chromaffin cells in the -/D group remained under 20% until 7 DIV (Figs. 2, 3), but on 14 DIV was apparently increased compared with those on 2, 5 and 7 DIV (p<0.01). The percentages of VIP-immunoreactive cells in the N/- group were always larger than those in the -/D group throughout the culture period. The differences in the percentage of VIP-immunoreactive cells between N/- and -/D groups were significant on 5 and 7 DIV (p<0.01). Morphological changes induced by NGF were not correlated with the changes in the proportion of VIP-immunopositive chromaffin cells (Spearman rank correlation test, p=0.08). The increase in the number of process-bearing cells by NGF was much more rapid than that of VIP-immunoreactive cells.

**Choline acetyltransferase (ChAT)**

Changes in the proportion of ChAT-immunopositive chromaffin cells were similar to those of VIP-immunoreactive cells. In the N/- group, the percentage of ChAT-immunoreactive cells among those immunopositive for TH increased gradually during the culture period, although in the -/D group the percentage of ChAT-immunopositive chromaffin cells was low and showed no marked increase (Figs. 4, 5). The percentages of ChAT-immunoreactive cells in the N/- group were significantly greater than those in the -/D group throughout the culture period (p<0.01). Morphological changes induced by NGF were not correlated with the changes in the proportion of ChAT-immunopositive chromaffin cells (Spearman rank correlation test, p=0.08). The increase in the number of process-bearing cells was much more rapid than that of ChAT-immunoreactive cells.

**Effect of depolarizing state**

Depolarizing concentrations of potassium chloride...
Fig. 1. TH- and NPY-immunoreactive cultured chromaffin cells at 7 DIV in NGF-treated (A–C), or DEX-treated (D–F) groups. A and D, Nomarski modulation contrast micrographs. B and E, TH-immunofluorescence staining micrographs. C and F, NPY-immunofluorescence staining micrographs. A–F: ×450
Fig. 2 A. Graphic representation of changes in the percentage of NPY-immunoreactive (NPY+) cells that are TH-immunoreactive (TH+) from 2 DIV to 14 DIV observed in a medium containing NGF (N/-) and in a medium containing DEX (-/D). Note the significant difference on 14 DIV between the N/- and -/D groups (Mann-Whitney U test, \( p < 0.05 \)). B. Graphic representation of changes in the percentage of process-bearing cells that are TH+ in the same cultures. The values represent the means of eight coverslips; error bars represent S.E.M.
Fig. 3. TH- and VIP-immunoreactive cultured chromaffin cells at 7 DIV in NGF-treated (A-C), or DEX-treated (D-F) groups. A and D. Nomarski modulation contrast micrographs. B and E. TH-immunofluorescence staining micrographs. C and F. VIP-immunofluorescence staining micrographs. A-F: ×450
Fig. 4 A. Graphic representation of changes in the percentage of VIP-immunoreactive (VIP+) cells that are TH-immunoreactive (TH+) from 2 DIV to 14 DIV observed in a medium containing NGF (N/-) and in a medium containing DEX (-/D). Note the significant differences in the percentage of VIP-immunoreactive cells on 5 and on 7 DIV between the N/- and -/D groups (Mann-Whitney U test, *p<0.01). B. Graphic representation of changes in the percentage of process-bearing cells that are TH+ in the same cultures. The values represent the means of eight coverslips; error bars represent S.E.M.
Fig. 5. TH- and ChAT-immunoreactive cultured chromaffin cells at 14 DIV in NGF-treated (A-C), or DEX-treated (D-F) groups. A and D. Nomarski modulation contrast micrographs. B and E. TH-immunofluorescence staining micrographs. C and F. ChAT-immunofluorescence staining micrographs. The cell bodies at 14 DIV generally became larger than those at 7 DIV. ×450
Fig. 6 A. Graphic representation of changes in the percentage of ChAT-immunoreactive (ChAT+) cells that are TH-immunoreactive (TH+) from 2 DIV to 14 DIV observed in a medium containing NGF (N/-) and in a medium containing DEX (-/D). The percentages of ChAT-immunoreactive cells in the N/- group are significantly greater than those in the -/D group throughout the culture period (Mann-Whitney U test, *p < 0.01). B. Graphic representation of changes in the percentage of process-bearing that are TH+ in the same cultures. The values represent the means of eight coverslips; error bars represent S.E.M.
Fig. 7 A. Graphic representation of changes in the percentage of VIP-immunoreactive (VIP+) cells that are TH-immunoreactive (TH+) at 2 DIV observed in a medium containing NGF (N/-) and in a medium containing DEX (-/D) with / without potassium chloride (KCl+ / KCl-). B. Graphic representation of changes in the percentage of process-bearing cells that are TH+ in the same cultures. The values represent the means of four coverslips; error bars represent S.E.M.
Phenotypic Alterations of NPY, VIP and ChAT

Fig. 8 A. Graphic representation of changes in the percentage of ChAT-immunoreactive (ChAT+) cells that are TH-immunoreactive (TH+) at 2 DIV observed in a medium containing NGF (N/-) and in a medium containing DEX (-/D) with/without potassium chloride (KCl+/KCl-). B. Graphic representation of changes in the percentage of process-bearing cells that are TH+ in the same cultures. The values represent the means of four coverslips; error bars represent S.E.M.
had no effect on the percentages of neuropeptide-positive or ChAT-positive chromaffin cells. There were no effects of the depolarization agents on the expression of VIP or ChAT, or the percentage of process-bearing cells (Figs. 7, 8). Percentages of NPY-immunopositive cells among those immunopositive for TH and that of process-bearing cells were unaffected with or without KCl (data not shown).

**DISCUSSION**

Neuronal form and function are closely related since the shape of a neuron determines its connections with other cells and also strongly influences the mechanism by which it processes synaptic information (Purves, 1988). Recent studies have shown that peptide growth factors and steroid hormones play an important role in shaping developmental decisions in neurons as well as chromaffin cells (Patterson and Nawa, 1993; Kawata, 1995).

In this study, NGF continued to increase the percentage of process-bearing cells during the 14 days in rat chromaffin cell culture. Most NGF-treated cultured chromaffin cells on 14 DIV presented the morphological appearance of mature sympathetic neurons. These cells were thought to have transdifferentiated into sympathetic neurons (Doupe et al., 1985a). DEX-treated cultured chromaffin cells did not change morphologically during the culture period. The conditions in DEX-treated cultures resembled the environment around chromaffin cells in vivo, which are surrounded by the adrenal cortex.

With our culture method, the total number of cells per coverslip did not obviously change during the culture period. In particular, there were no significant differences in the number of chromaffin cells between the NGF-treated and the DEX-treated groups throughout the culture period. Although NGF showed mitogenic activity for immature normal chromaffin cells and several portions of chromaffin cells were labeled by \(^{3}H\) thymidine (Lilien and Claude, 1985; Herman et al., 1994), the number of cultured chromaffin cells did not increase as the culture proceeded under either NGF treatment or DEX treatment (Doupe et al., 1985a; the present study). It suggested that the number of the proliferated cells was not more than that of the lost cells during the culture period. In contrast, the number of chromaffin cells cultured without NGF and DEX was significantly decreased by 14 DIV compared with the NGF- or DEX-treated groups. This finding suggests that NGF or DEX is essential for the long-term culture of chromaffin cells at low density.

The percentages of NPY-immunopositive chromaffin cells did not change markedly whether treated with NGF or DEX throughout the culture period, and half of the cells retained NPY immunoreactivity. These proportions were nearly the same as those previously reported in vivo (Henion and Landis, 1990; Tyrrell and Landis, 1994). The percentages of NPY-positive cells among sympathetic neurons and of NPY-immunopositive chromaffin cells in postnatal day 0 rats were estimated to be 57% and 40%, respectively. In the NGF-treated group, the percentage of NPY-immunopositive cells was decreased only on 14 DIV, compared with that following other periods in culture. Although the definitive reason remains unknown, the decrease in the percentage of NPY-immunopositive cells might have been affected by the relative increase in the number of VIP-immunopositive cells on 14 DIV. Some chromaffin cells might be changed from NPY-positive to VIP-positive cells.

The proportion of VIP-immunopositive chromaffin cells was gradually increased from 2 DIV to 14 DIV in the NGF-treated group and was also raised at 14 DIV in the DEX-treated group. ChAT-immunopositive cells in both groups showed the same tendency as those immunopositive for VIP. In vivo, the numbers of VIP- and ChAT-immunopositive chromaffin cells in rat adrenal glands are very low (Kondo, 1985). The nature of the signal responsible for the increase in the number of VIP- or ChAT-immunopositive chromaffin cells in vitro remains unclear. One possibility is that the chromaffin cells became transiently immature sympathetic neurons when they were induced to transdifferentiate by NGF. Immature sympathetic neurons, comprising about 15% of sympathetic neuroblasts in embryonic day-16.5 rats, were VIP-immunopositive (Tyrrell and Landis, 1994). Another possibility is that chromaffin cells themselves or other non-chromaffin cells might produce the factors which induced the VIP and cholinergic properties in culture. In the axotomized superior cervical ganglion (SCG) in vivo or in organ-cultured SCG, leukemia inhibitory factor (LIF)-like factors that were thought to originate in non-neuronal cells induced the expression of VIP in ganglion cells (Zigmond et al., 1992; Hyatt-Sachs et al., 1993). The latter possibility was thought to be more likely because, in the DEX-treated group, the percentages of VIP-immunopositive and ChAT-immunopositive chromaffin cells were low from 2 DIV to 7 DIV, but on 14 DIV both showed apparent increases.

In the present study, we did not focus on the determination of factors which induced the phenotypic alterations of cultured chromaffin cells. Al-
though there were few non-chromaffin cells in this experiment, the possibility that unknown factors which originated from those cells might affect the phenotypic changes should not be excluded. Further investigations will be necessary to determine these factors.

Most importantly, the morphological changes showed no significant correlation with the alterations in the proportions of NPY-, VIP- or ChAT-immunopositive chromaffin cells, although the percentages of VIP- or ChAT-immunopositive chromaffin cells in the NGF-treated group showed much greater increases than those in the DEX-treated group. This suggests that NGF might accelerate the phenotypic changes evoked by as yet unknown factors.

The potassium chloride-containing medium mimicked depolarizing conditions in vivo (DOUPE et al., 1985a). If the alterations in cultured chromaffin cells were induced by the removal of preganglionic nerve fibers, the high concentration of potassium chloride would inhibit them because of the maintenance of the depolarizing state. The addition of potassium chloride in this study had no effect on the induction of VIP- or ChAT-immunoreactive chromaffin cells in vitro. These results suggest that they were not induced by the loss of preganglionic innervation in culture.

REFERENCES


Dr. Masafumi Morimoto
Department of Anatomy and Neurobiology
Kyoto Prefectural University of Medicine
Kawaramachi-Hirokoji, Kamigyo-ku
Kyoto, 602 Japan

森本 昌史
602 京都市上京区河原町通広小路
京都府立医科大学
第一解剖学教室