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Long Lasting Increase in Neuropeptide Y Gene Expression in Rat Adrenal Gland with Reserpine Treatment: Positive Regulation of Transsynaptic Activation and Membrane Depolarization

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SUMMARY

To elucidate how the neuropeptide Y (NPY) gene is regulated by physiological/pharmacological changes in neural functions, the expression and regulation of the NPY gene were studied by measuring changes in the abundances of NPY and NPY mRNA in the adrenal gland and brain regions of rats *in vivo* and in PC12 rat pheochromocytoma cells after reserpine treatment. Long term treatment with reserpine *in vivo*, which causes hypotension and increased splanchnic nerve activity, induced prolonged increases in the abundance of NPY mRNA and putative NPY pre-mRNA, with concomitant increases in NPY, in the adrenal gland in a tissue-dependent manner but caused no changes in the abundance of β-actin mRNA. Transection of the splanchnic nerves almost completely (76%) prevented the reserpine-induced increases in the abundance of NPY mRNA and NPY pre-mRNA, but denervation alone did not affect their steady state levels. These results suggested that increased activity of the splanchnic nerves regulates NPY gene expression positively in the adrenal gland, probably at the level of transcription. In PC12 cells, reserpine decreased the abundance of NPY mRNA directly, but nicotinic receptor activation increased its abundance transiently and the persistent membrane depolarization increased its abundance markedly. Thus, NPY gene expression is positively regulated by membrane depolarization via increased transsynaptic activation with reserpine.

NPY, one of the most abundant neuropeptides, is widely distributed in the central and peripheral nervous systems and is an important neuromodulator/cotransmitter in catecholaminergic neurons as well as in the adrenal gland, which is derived from sympathetic neurons (1–6).

NPY has essential functions in neural transmission, including nonadrenergic sympathetic neurotransmission and higher central cognition function via modulation of the release of several transmitters and hypothalamic hormones, and NPY also has direct postsynaptic actions through specific NPY receptors (7–9). For example, NPY is involved in the regulation of peripheral and cardiac arterial blood pressure and cardiac functions (8, 10–13). Central administration of NPY causes hypotension, whereas its peripheral administration produces a prolonged pressor response (8, 11, 14). Brief reviews of NPY neuronal systems and functions have been published (15, 16).

There is increasing evidence that the level of NPY is regulated by physiological factors, such as aging, innervation, stress, and treatments with pharmacological agents that influence the blood pressure (17–22). Reserpine, an antihypertensive drug, and other pharmacological agents have been found to cause tissue-specific changes in the steady state NPY contents in some organs related to vascular control (19, 20, 23–27). These findings are consistent with the idea that the system regulating NPY biosynthesis/turnover is important for physiological modulation of neural functions *in vivo*. However, although NPY is an important constituent of sympathetic neurons, much less is known about the regulation of NPY biosynthesis and turnover than about the systems regulating catecholamines, including the gene expression of TH.

Molecular biological studies have been done to investigate the expression and regulation of NPY peptides, and the structures of prepro-NPY and NPY precursor gene (NPY gene) in mammalian species have been determined by cDNA and genomic cloning and sequencing (28–30). Moreover, cloned cDNA or oligonucleotide probes have been used to obtain information about the regulation of NPY gene expression (30–36). Results from the *in situ* hybridization technique have shown that reserpine increases the abundance of NPY precursor mRNA (NPY mRNA) in sympathetic ganglia and the adrenal glands (34, 35, 37).

ABBREVIATIONS: NPY, neuropeptide Y; HPLC, high performance liquid chromatography; SD, Sprague-Dawley; NPY-LI, neuropeptide Y-like immunoreactivity; TH, tyrosine hydroxylase; ENK, enkephalin; bp, base pairs; kb, kilobases.

To elucidate how the NPY gene is regulated by physiological and pharmacological changes of neural functions and, especially, how antihypertensive drugs can modify blood pressure via changes in gene expression of vasoconstrictive NPY peptides, the effects of reserpine on NPY gene expression in a sympathetic organ, the adrenal gland, and in regions of the brain were examined by measuring the abundance of NPY and NPY mRNA by quantitative Northern blot analysis. For this purpose we used a cloned rat NPY cDNA probe and radioimmunoassay with specific NPY antiserum. We found that reserpine induced tissue-specific increases in NPY gene expression in the rat adrenal via transsynaptic control, with concomitant increases in NPY. We also investigated the direct effects of reserpine and depolarization signals on NPY gene expression in PC12 rat pheochromocytoma cells, to clarify the molecular mechanisms involved. Preliminary reports of our results have appeared in abstract form (38, 39).

Materials and Methods

Drug treatment. Male SD rats were used in all experiments. Reserpine (0.5 mg/kg of body weight) in saline was injected intraperitoneally into 8-week-old rats once a day for 5 days, whereas control rats received the same volume (0.1 ml) of saline as a vehicle. The rats were killed by decapitation 24 hr after the last injection.

Adrenal denervation. Male SD rats, 7 weeks old, were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and the nerve fibers leading from the main splanchnic trunk to the left adrenal gland were transected. Particular care was taken not to damage the blood vessels supplying the adrenal. The rats were treated with reserpine or vehicle, as described above, from 1 week after the operation.

Cell culture. PC12 cells, obtained from Dr. Gordon Guroff (National Institutes of Health, Bethesda, MD), were cultured at 37° in 75- or 175-cm^2 flasks in 85% Dulbecco's modified Eagle's medium, 10% heat-inactivated horse serum, 5% heat-inactivated fetal calf serum, under an atmosphere of 90% air/10% carbon dioxide. Stock solutions of reserpine, nicotine, and hexamethonium (×500 to ×1000) were prepared in saline. Cells exposed to the stated concentrations of these compounds appeared morphologically healthy and continued to divide.

Peptide extraction and radioimmunoassay. Rats were decapitated and regions of their brains, dissected according to the method of Glowinski and Iversen (40), and their adrenal glands were promptly removed and homogenized in a mixture of 1 M acetic acid and 95% ethanol (20:80 v/v), in polypropylene tubes, with a Hiscotron. Brain homogenates were prepared in 10 volumes of the mixture and adrenal homogenates in 1 ml/pair of adrenals. The homogenates were sonicated and then centrifuged at 17,700 × *g* for 10 min at 4°, and the resulting pellet was reextracted with the same acidified ethanol. The supernatants were combined and dried in a vacuum concentrator, and the dried extracts were dissolved in 1 ml of H_2O and centrifuged at 12,300 × *g* for 10 min at 2°. NPY immunoreactivity in the clear supernatant was then measured by radioimmunoassay, using rat NPY (or human NPY) as a standard, as described before (17, 18).

Characterization of NPY immunoreactivity by reverse phase HPLC. For characterization of the NPY immunoreactivity by reverse phase HPLC, the supernatants were applied to an octadecylsilyl silica cartridge (Sep-Pak C_{18}). The cartridge was then washed with 20 ml of H_2O, and the adsorbed NPY immunoreactivity was eluted with 4 ml of 60% acetonitrile containing 0.1% trifluoroacetic acid. Samples obtained from the Sep-Pak C_{18} cartridge were lyophilized, dissolved in 0.2 ml of H_2O, and centrifuged at 14,900 × *g* for 10 min. Then, 0.1 ml of the supernatant was applied to a HPLC BioSil ODS 10 column (4 × 250 mm). Material on this reverse phase column was eluted with a linear gradient of 20 to 60% acetonitrile, containing 0.1% trifluoroacetic acid, over a period of 60 min at a flow rate of 1 ml/min. Fractions (1 ml) of eluate were collected, lyophilized, and tested for NPY immunoreactivity. Rat NPY and porcine peptide YY were used as standard markers.

Preparation of cellular RNA. Regions of the brain were dissected out, as described by Glowinski and Iversen (40). Pairs of adrenal glands and tissues from various regions of the brain, obtained immediately after decapitation, were homogenized in at least 5 volumes of 4 M guanidinium thiocyanate solution. Cultured cells in single 175-cm^2 flasks were treated in the same way. Then, the total cellular RNAs in the extracts were purified by centrifugation through 5.7 M CsCl (41) and quantitated by measurement of their absorbances at 260 nm.

Quantitation of NPY mRNA. NPY mRNA abundance was measured by Northern blot analysis and comparison of autoradiographic signals with those of coelectrophoresed standard RNA samples. In practice, RNA (25–50 μg) was denatured in 50% formamide, 6% formaldehyde, for 10 min at 60° and separated by electrophoresis in a 1.2% agarose gel containing 6% formaldehyde and 0.5 μg/ml ethidium bromide. The RNA was then transferred to a Nytran nylon membrane and hybridized with the 511-bp *Eco*RI insert of clone rNPY2, nick-translated with [α-^{32}P]dCTP, as described previously (30). For standardization, at least five different amounts (2.5–100 pg) of pBL-NPY1 transcripts or a standard rat striatum RNA preparation were run simultaneously, with or without carrier rat liver RNA (30). NPY mRNA autoradiographic signals were quantitated by scanning densitometry and integration of peak areas corresponding to mature NPY mRNA (approximately 800 bases). Putative NPY pre-mRNA bands were not quantitated. Northern blot rather than dot blot analysis was used because the latter method was usually not sufficiently sensitive for quantitation of NPY mRNA (30).

Quantitation of β-actin mRNA. RNA (25–50 μg) prepared from rats treated with reserpine or vehicle was transferred to Nytran nylon membranes, and the membranes were hybridized with the nick-translated 700-bp *Eco*RI-*Hin*dIII insert of a plasmid containing rat β-actin cDNA in pUC8 (42). The abundance of β-actin mRNA in extracts of the adrenal gland, striatum, and medulla oblongata plus pons of rats treated with reserpine was then determined by quantitative Northern blot analysis and comparison of their autoradiographic signals with those of coelectrophoresed samples from control animals.

Statistical methods. Statistical significance was determined by Student's *t* test.

Materials. Rat NPY (human NPY) was obtained from Peninsula Laboratories; for HPLC, BioSil ODS 10 columns (250 × 4mm) were obtained from Bio-Rad Laboratories. Reserpine (Daiichiseiyaku Co.), nicotine (Nakarai Chemical, Ltd.), veratridine (Sigma), and hexamethonium chloride (Wako Pure Chemical) were also used.

Results

Reserpine-induced biphasic changes in the level of NPY in the adrenal gland. Intraperitoneal injection of 0.5 mg/kg reserpine into SD rats resulted in a rapid initial decrease of NPY-LI in the adrenal gland, to a minimum of 55% of the control level after 24 hr, significantly without any change in the NPY mRNA abundance (Fig. 1; see also Fig. 6A). Then, the level of NPY-LI gradually increased, reaching 210% of that in vehicle-treated controls on day 3 after the first injection. This increased level of NPY-LI was maintained for 14 days after the end of reserpine treatment (Fig. 1).

The NPY-LI levels in various regions of the brain (cerebral cortex, striatum, and medulla oblongata plus pons), measured as controls, showed no significant changes during or after reserpine treatment (Fig. 2).

NPY-LI extracted from the adrenal glands and regions of the brain of rats was subjected to reverse phase HPLC and was characterized as described in Materials and Methods. Fig. 3 shows typical HPLC chromatographic patterns obtained with extracts of rat adrenal gland and cerebral cortex, indicating

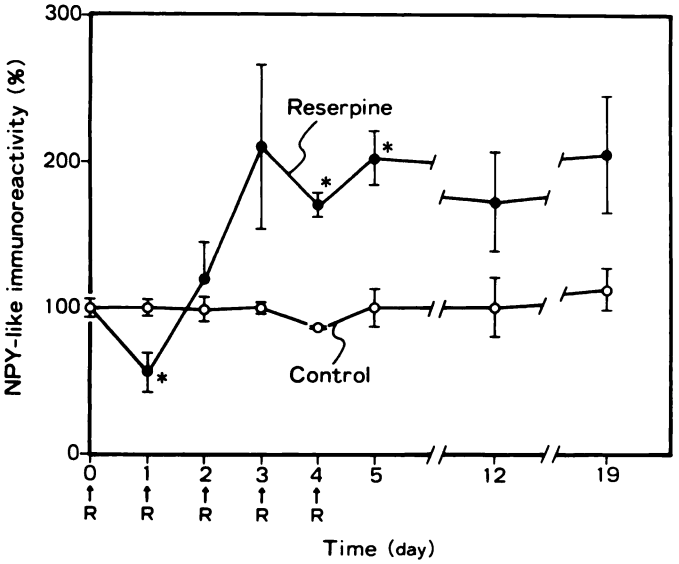

Fig. 1. Time course of changes in the NPY immunoreactivity contents of rat adrenal gland following injection of reserpine. Eight-week-old male SD rats were treated with reserpine (0.5 mg/kg, intraperitoneally) (*R*) or vehicle once a day. The adrenal glands were immediately dissected at the various days, and the NPY immunoreactivity was extracted from the adrenal glands and quantitated as described in Materials and Methods. Results are expressed as percentages of untreated (0-day, 8-week-old) values. Each *point* is the mean ± standard error for animals in comparison with a control group of animals killed at the start of the experiments (n = 10–17). The mean control value ± standard error of the 0-day group was 80 ± 5 pmol/g of tissue (n = 17) (adrenal NPY cotnent). *, Significantly different from the control value, $p < 0.05$.

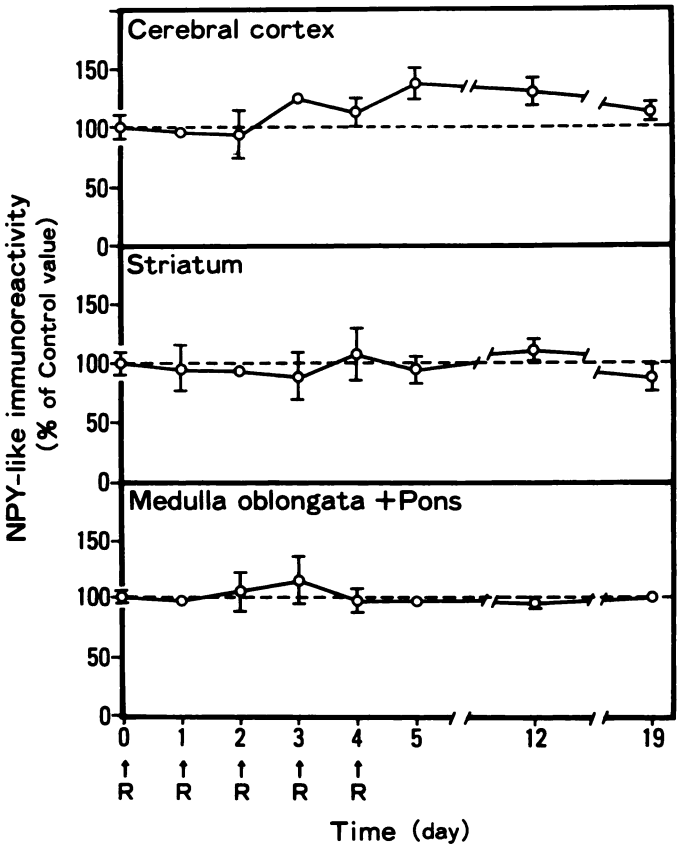

Fig. 2. Absence of changes in the NPY immunoreactivity contents of rat brain regions following injection of reserpine (*R*). Results are expressed as percentages of control values. Each *point* is the mean ± standard error for eight animals in comparison with a control group of the same number of animals killed on the same day. The data did not show statistically significant changes. The mean values ± standard errors for control (8-week-old male) rats were: cerebral cortex, 91 ± 9 pmol/g of tissue; striatum, 73 ± 7 pmol/g of tissue; and medulla oblongata plus pons, 26 ± 1 pmol/g of tissue.

only one peak with the same retention time as the rat NPY standard in each case for which the HPLC analysis was performed. These results indicate that the NPY-LI consists of NPY itself.

Association of reserpine-induced increases in NPY levels with increases in NPY gene expression in the adrenal gland. Changes in the level of NPY in the adrenal gland might be due to changes in turnover (and/or release) of NPY peptides and/or in biosynthesis, including gene expression, of the NPY precursor. To examine these possibilities, we measured the NPY mRNA abundance in the adrenal gland and brain regions of rats treated with reserpine.

A reported method for RNA extraction (41) was found to yield high constant recovery without causing any detectable degradation of RNA. The RNA yields per rat were not affected by reserpine treatment and were as follows: pair of adrenal glands, 185 ± 15 μg (n = 31); cerebral cortex, 640 ± 50 μg (n = 10); striatum, 129 ± 7 μg (n = 10); and medulla oblongata plus pons, 89 ± 9 μg (n = 21).

The mRNA abundance for NPY precursor was determined by quantitative Northern blot analysis using samples of 25 μg of total cellular RNA and a specific probe. The 511-bp rat NPY cDNA probe hybridized with three mRNA species of different sizes (Figs. 4 and 5). In addition to mature NPY mRNA (800 bases), larger RNA species (3300 and 700 bases) were also observed in samples from rat adrenal gland and various regions of the brain. These larger RNA species, although not characterized further, correspond in size to unspliced or incompletely spliced transcripts predicted from the structure of the rat NPY gene (29, 30, 36). These species are likely to be NPY pre-mRNA species, because they are changed to the same extent as mature NPY mRNA by various treatments, such as nerve growth factor, glucocorticoids, and forskolin (36).

Treatment with 0.5 mg/kg reserpine clearly increased the levels of mature NPY mRNA and the putative NPY pre-mRNA species to equal extents and this change was specific to the adrenal gland, suggesting that long term (5 days) treatment with reserpine caused transcriptional activation (Figs. 4 and 5). Table 1 summarizes results on reserpine-induced changes in the NPY mRNA abundance. In contrast to its effects on the adrenal glands, reserpine did not influence the levels of mRNA or putative pre-NPY mRNA in various regions of the brain (Figs. 4 and 6B and Table 1). Thus, reserpine increased NPY gene expression in the adrenal gland specifically.

The time course of increase in the NPY mRNA abundance in the adrenal gland induced with reserpine is shown in Fig. 6A. The increase was observed from day 2 of reserpine treatment and enlarged gradually until 5 days after the first injection. The maximum increase on day 5 was 470 ± 40% of the value of the untreated group on day 0 and 290 ± 20% of that of the vehicle-treated controls on day 5. The NPY mRNA abundance remained significantly increased for at least 7 days after the end of reserpine treatment. This means that reserpine caused prolonged elevation of NPY gene expression.

Interestingly, the NPY mRNA level in the adrenal gland of

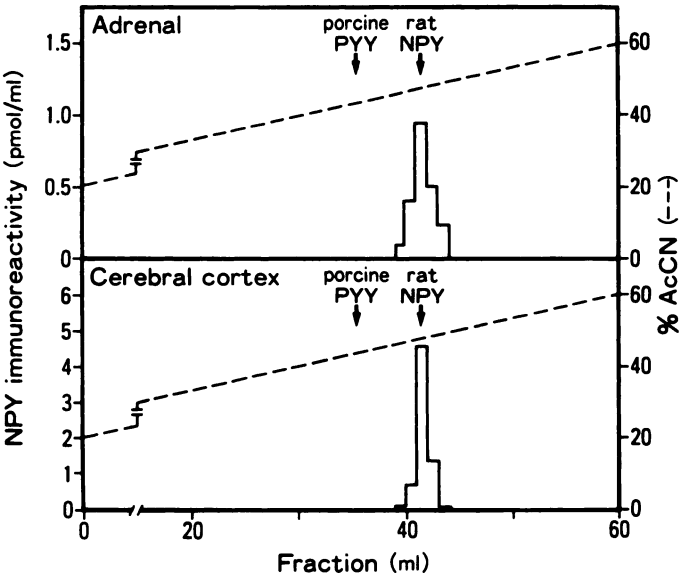

Fig. 3. HPLC analysis of NPY immunoreactivity in rat adrenal glands and brain. The acetic acid/ethanol extracts of tissues were lyophilized, redissolved in water, and passed through an octadecylsilyl silica cartridge (Sep-Pak C_{18}; Waters Associates) in water. Peptides were eluted with 4 ml of 60% acetonitrile containing 0.1% trifluoroacetic acid. The samples were lyophilized, dissolved in 0.2 ml of water, and centrifuged at 15,000 × *g* for 10 min. The supernatant fraction (0.1 ml containing 1.9 pmol of NPY immunoreactivity) was applied to a reverse phase BioSil ODS 10 column (4 × 250 mm). The column was developed with a linear gradient of 20–60% acetonitrile (*AcCN*) in 0.1% trifluoroacetic acid over a period of 60 min, at a flow rate of 1 ml/min. One-milliliter fractions were collected, lyophilized, reconstituted in water, and radioimmunoassayed to determine NPY content. The recovery of standard NPY on the HPLC column was 91%. *Arrows*, elution positions of synthetic rat (human) NPY and porcine peptide YY (*PYY*).

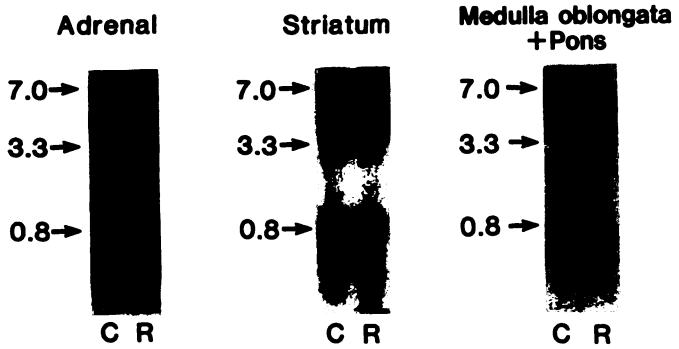

Fig. 4. Northern blot analysis of NPY mRNA from various tissues following reserpine treatment. Eight-week-old male rats were treated with reserpine (0.5 mg/kg, intraperitoneally) (*R*) or vehicle (*C*) once a day for 5 days. Tissues were immediately dissected and lysed in 5–10 volumes of 4 M guanidinium thiocyanate solution. Total cellular RNA was prepared and purified by centrifugation through 5.7 M CsCl (41) and was quantitated by absorbance at 260 nm. Total cellular RNA (25 μg/sample) from rat adrenal gland, striatum, and medulla oblongata plus pons was electrophoresed in a formaldehyde-agarose gel, blotted, and hybridized with the 511-bp *Eco*RI insert of the pBL-NPY1 plasmid. *Numbers* at the *left*, lengths of hybridized RNA (0.8, 3.3, and 7.0 kb). Sources of RNA are shown above the lanes.

vehicle-treated controls also increased, reaching 180 ± 5% of the initial value by day 3. Thus, the stress of vehicle administration alone induced NPY gene expression in the adrenal gland.

Absence of effect of reserpine on β-actin gene expression. In accordance with previous findings (42), the rat β-actin cDNA probe hybridized with a single mRNA species of 2200 bases for β-actin mRNA in the adrenal gland and brain of rats (Fig. 7). Reserpine treatment (5 days, 0.5 mg/kg, intraperitoneally, once a day) did not change the abundance of mRNA for this constitutive gene in either the adrenal gland or the brain regions (Fig. 7 and Table 2). These results indicate that reserpine had a specific effect on NPY gene expression in the adrenal gland.

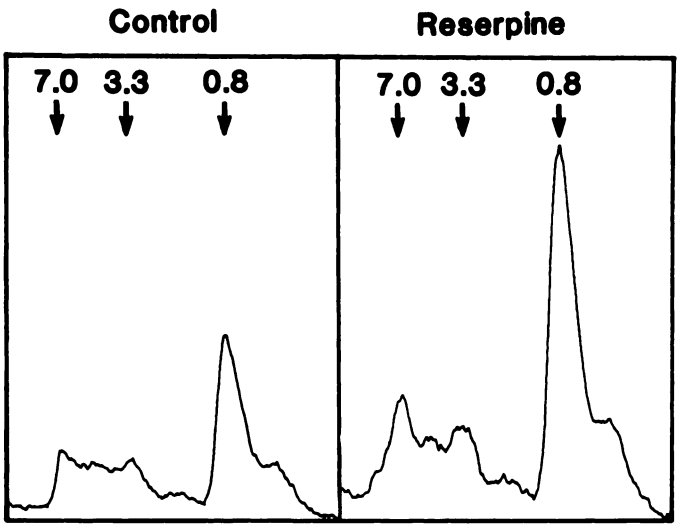

Fig. 5. Densitogram of reserpine-induced changes in NPY mRNA of rat adrenal glands. NPY mRNA autoradiographic signals of the adrenal gland of rats treated with reserpine or vehicle were quantitated with a scanning densitometer (Shimadzu model CS-930). Exposure times of autoradiograms were chosen so that peak areas were within the range of linear variation with amount of RNA applied. *Numbers* above the *traces*, the lengths of hybridized RNA (0.8, 3.3, and 7.0 kb).

TABLE 1

Reserpine-induced changes in the NPY mRNA content of rat adrenal gland and brain

Eight-week-old male rats were treated with reserpine (0.5 mg/kg, intraperitoneally) or vehicle once a day for 5 days. Then, tissues were immediately dissected and their NPY mRNA contents were quantitated, as described in Materials and Methods. Values are mean ± standard error of the number of experiments in parentheses.

Region	NPY mRNA abundance	
	Control	Reserpine
	pg/μg of total cellular RNA	
Adrenal	1.7 ± 0.1 (4)	4.2 ± 0.6[a] (4)
Cerebral cortex	3.7 ± 0.3 (4)	2.5 ± 0.2 (4)
Striatum	6.1 ± 0.04 (3)	5.5 ± 1.4 (3)
Medulla oblongata + pons	0.94 ± 0.06 (3)	0.96 ± 0.06 (3)

[a] Significantly different from the control value, $p < 0.01$.

Effects of splanchnic nerve transection on NPY gene expression in the adrenal glands. To determine whether the reserpine-induced increase in the level of NPY mRNA was due to a direct effect on chromaffin cells, we examined the effects of this drug on the NPY mRNA level in denervated rats (Fig. 8). The left splanchnic nerve was transected and a sham operation was made on the right side. Twelve days later, the NPY mRNA level in the left adrenal gland was not significantly different from that in the right adrenal gland. However, splanchnic transection resulted in 76% inhibition of the reserpine-induced increase in the NPY mRNA level and simultaneous inhibition of the increase in the putative NPY pre-

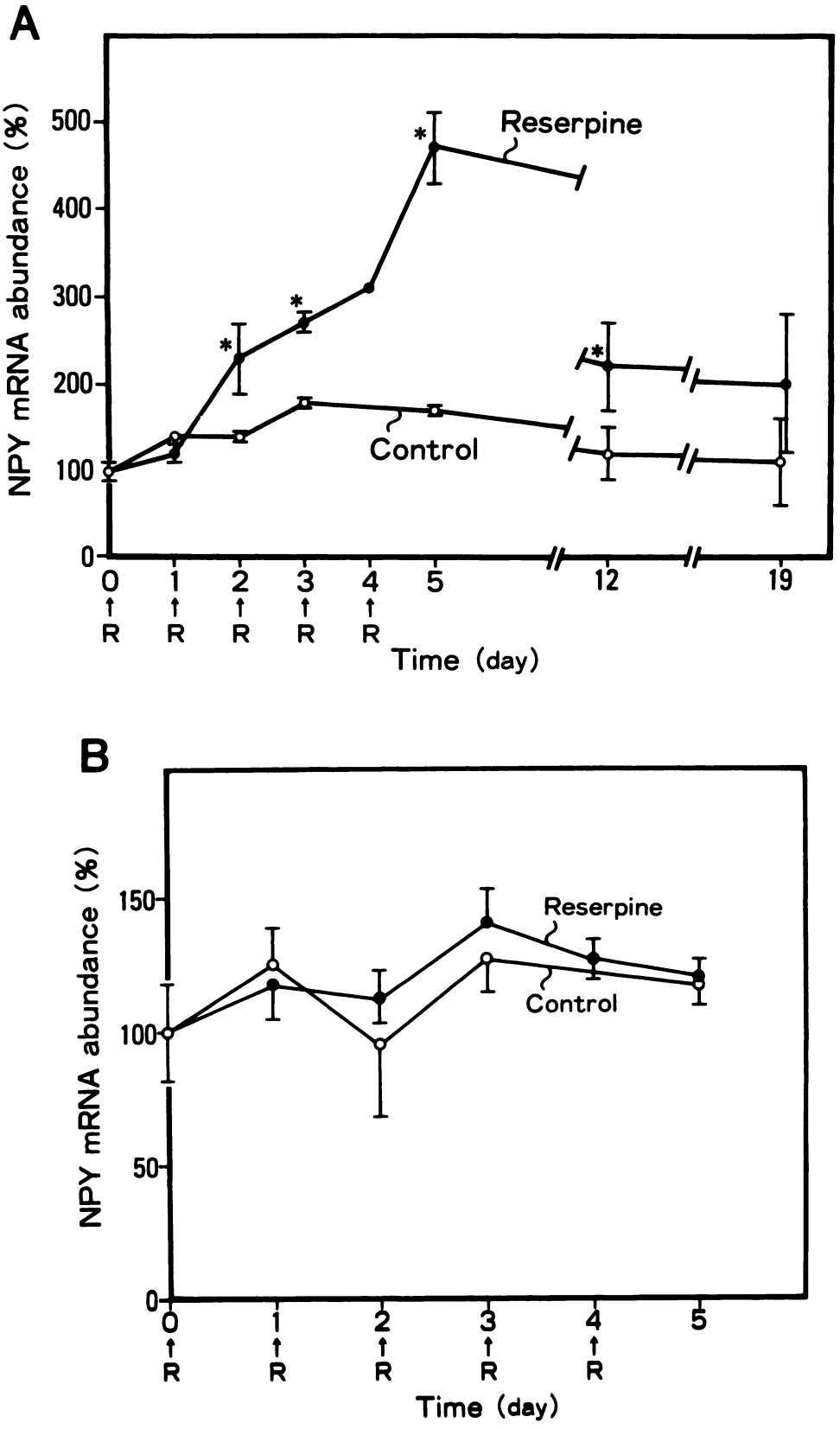

Fig. 6. Time course of changes in NPY mRNA levels in the adrenal gland and medulla oblongata plus pons following reserpine treatment. Eight-week-old male rats were treated with reserpine (0.5 mg/kg, intraperitoneally) (*R*) or vehicle once a day for 5 days. The tissues (adrenals and medulla oblongata plus pons) were immediately dissected at the various times, and the total cellular RNA was purified from the tissues by the method of Chirgwin (41) and quantitated. The NPY mRNA amounts were quantitated by Northern blot analysis, as described in Materials and Methods. Data are mean ± standard error of three to five independent experiments. Control values (at 0 day) were 1.1 ± 0.1 pg/μg of total cellular RNA for the adrenal gland and 0.8 ± 0.1 pg/μg of total cellular RNA for the medulla oblongata plus pons. *, Significantly different from the control value, $p < 0.01$. A, Adrenal gland; B, medulla oblongata plus pons.

mRNA species. These results showed that reserpine induced increases in the NPY mRNA abundance by a neurogenic mechanism. Moreover, because denervation alone did not affect the NPY mRNA level in the adrenal gland under basal conditions, the activity of the afferent splanchnic nerve does not seem to affect NPY gene expression in the adrenal gland under unstimulated conditions.

Effects of receptor activation and membrane depolarization on NPY gene expression in PC12 rat pheochromocytoma cells. The normal counterpart to PC12 pheochro-

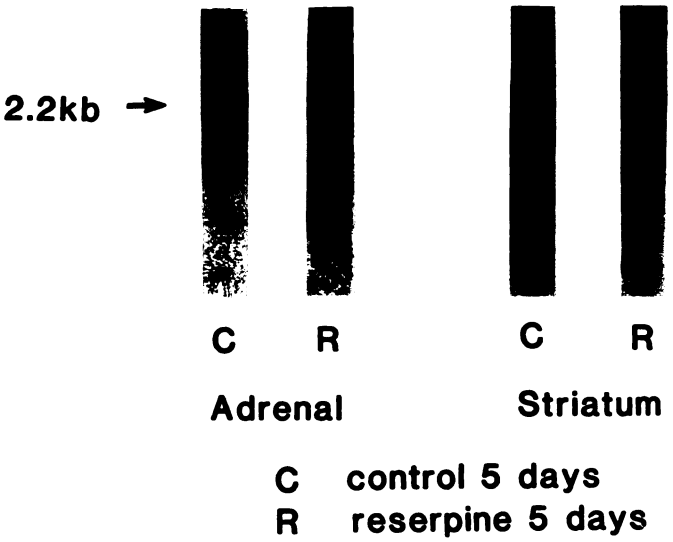

Fig. 7. Northern blot analysis of hybridizable β-actin mRNA in rat adrenal gland and brain striatum. Total cellular RNA (25 μg) isolated from rats treated as described in the legend to Fig. 4 was subjected to electrophoresis in a formaldehyde-agarose gel, transferred to nylon filters, and then hybridized with the nick-translated ^{32}P-labeled β-actin cDNA probe, as described in Materials and Methods. A single hybridizable β-actin mRNA band was visualized by autoradiography and quantitated. The *number* at the *left*, the length of β-actin mRNA (2.2 kb).

TABLE 2

Absence of effect of reserpine treatment on the β-actin mRNA abundance in the adrenal gland, striatum, and medulla oblongata plus pons

Samples of total cellular RNA identical to those of rats treated with reserpine (0.5 mg/kg, intraperitoneally) or vehicle for 5 days for Fig. 4 were used for quantitative Northern blot analysis of β-actin mRNA, as described in Materials and Methods. Values are expressed as percentages of those of control rats and represent mean ± standard error from three individual samples.

Region		β-Actin mRNA abundance
		% of control
Adrenal	Control	100 ± 5
	Reserpine	115 ± 14
Striatum	Control	100 ± 9
	Reserpine	97 ± 23
Medulla oblongata + pons	Control	100 ± 15
	Reserpine	86 ± 3

mocytoma cells is the chromaffin cell of the adrenal medulla, and NPY is expressed primarily in the chromaffin cells in rat adrenal gland (5, 20, 33, 35). Therefore, we investigated the effects of reserpine, nicotine (receptor activation), and membrane depolarization on NPY gene expression in PC12 cells.

Untreated PC12 cells express a low level of NPY mRNA (800 bases; 0.16–0.51 pg/μg of total cellular RNA) and two lesser putative pre-NPY mRNA species (3300 and 7000 bases) (30, 36).

As shown in Table 3, reserpine (2.4 μg/ml, 4 μM, 5 days) caused a 66% decrease in the NPY mRNA abundance in PC12 cells, rather than increasing its level. This result supported the conclusion that the effect of reserpine in inducing increases in the NPY mRNA abundance in the adrenal gland is indirect.

To confirm the transsynaptic control of NPY gene expression in chromaffin cells by splanchnic nerve, we examined the effect of nicotine on the NPY mRNA abundance in PC12 cells. Treatment of the cells with 10 μM nicotine resulted in a slight and transient increase in the NPY mRNA abundance (by 88%

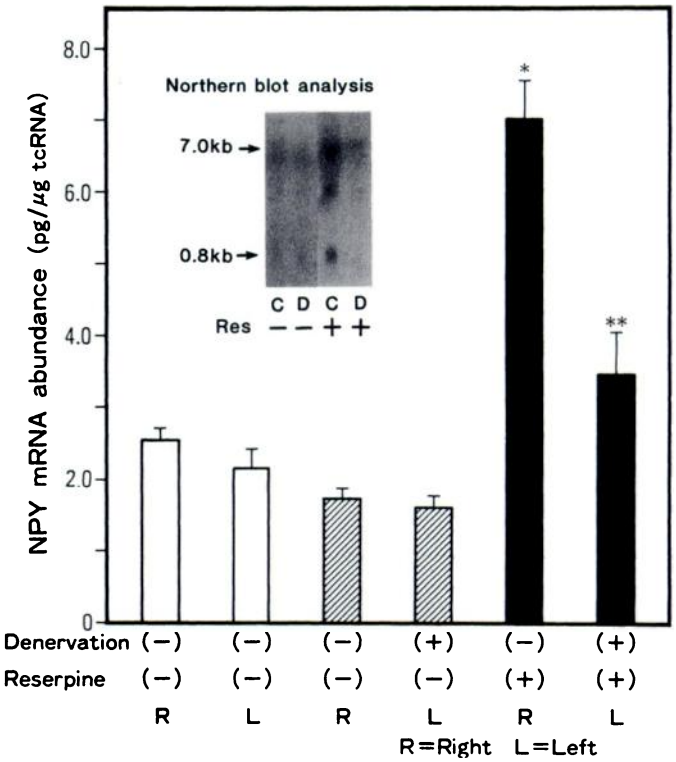

Fig. 8. Effect of splanchnic nerve transection on reserpine-induced increases in the NPY mRNA content. The splanchnic fibers leading from the main trunk to the left adrenal gland of 7-week-old rats were transected under pentobarbital anesthesia 1 week before the beginning of reserpine administration. The surgically treated animals were given 0.5 mg/kg reserpine or saline as a vehicle, intraperitoneally, once a day for 5 days before they were killed. The NPY mRNA contents of bilateral adrenal glands of untreated rats at the same age (8 weeks and 5 days old) were measured as a control. The columns represent mean ± standard error from four or five observations. Three adrenals of the ipsilateral side were combined for one measurement. *, Significantly different from the untreated group, $p < 0.01$; **, Significantly different from the sham-operated side, $p < 0.01$. *Inset*, Northern blot analysis of NPY mRNA from rat adrenal glands ($n = 5$) in control (*C*) and denervated (*D*) animals. Reserpine-induced increase in NPY mRNA content was abolished after denervation. *Numbers* at the *left*, lengths of hybridized RNAs. *Res*, reserpine; *R*, right side; *L*, left side.

TABLE 3

Effect of reserpine on the NPY mRNA abundance of PC12 rat pheochromocytoma cells

PC12 cells (70–80% confluent) were treated with 4 μM (2.4 μg/ml) reserpine or vehicle for 5 days. The abundance of NPY mRNA was determined by Northern blot analysis, as described in Materials and Methods. The data are mean ± standard error obtained from three independent samples.

Treatment		NPY mRNA abundance
		pg/μg of total cellular RNA
Control	5 days	0.51 ± 0.07
Reserpine	5 days	0.17 ± 0.04*

* Significantly different from the control value, $p < 0.01$.

after 8 hr), and this increase was blocked by hexamethonium (Fig. 9).

We also examined the effect of persistent depolarization of the membranes of PC12 cells on their NPY gene expression. As shown in Table 4, 24-hr treatment with 20 μM veratridine, an activation of voltage-sensitive sodium channels, increased the NPY mRNA level to 260% of that in control cells. This increase was completely blocked by 1 μM tetrodotoxin. High potassium (50 mM) medium also increased the NPY mRNA

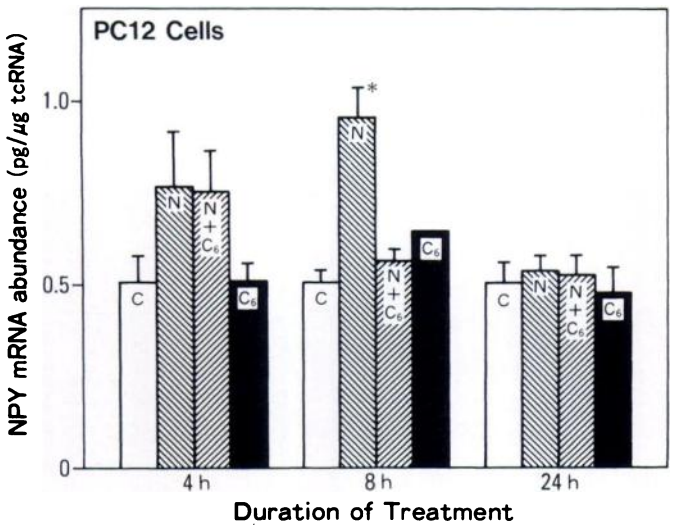

Fig. 9. Effect of nicotine on the NPY mRNA abundance of PC12 rat pheochromocytoma cells. PC12 cells (80% confluent) were incubated with 10 μM nicotine (*N*) and/or 50 μM hexamethonium (C_6) for the indicated durations. The quantitation of NPY mRNA abundance was performed by Northern blot analysis, as described in Materials and Methods. Data show mean ± standard error (two to four experiments). *, Significantly different from the control value, $p < 0.05$.

TABLE 4

Effect of membrane depolarization on the NPY mRNA abundance of PC12 rat pheochromocytoma cells

PC12 cells (60–70% confluent) were treated with the indicated agents for 24 hr (TTX, tetrodotoxin). The quantitation of NPY mRNA abundance in the cells was carried out as described for Table 3. Values are mean ± standard error for three independent samples.

Treatment	NPY mRNA abundance
	pg/μg of total cellular RNA
Control	0.16 ± 0.02
Veratridine (20 μM)	0.42 ± 0.02[a]
Veratridine (20 μM) + TTX (1 μM)	0.13 ± 0.01
TTX (1 μM)	0.20 ± 0.07
KCl (50 mM)	0.73 ± 0.01[a]
KCl (50 mM) + TTX (1 μM)	0.56 ± 0.14[b]
NaCl (50 mM)	0.27 ± 0.03

[a] Significantly different from the control value, $p < 0.01$.
[b] $p < 0.05$.

abundance to 460% of that of controls, and this increase was not blocked by tetrodotoxin. Thus, nicotinic receptor activation produces a slight transient increase in NPY gene expression, and persistent membrane depolarization results in a marked increase in NPY gene expression in PC12 rat pheochromocytoma cells.

Discussion

The recent finding that NPY coexists with catecholamines in sympathetic neurons and is coreleased with them on nerve stimulation suggests that NPY plays an important role as a cotransmitter/neuromodulator in sympathetic neurotransmission (4–6, 43). Reserpine, an antihypertensive drug, depletes sympathetic neurons of monoamines, including noradrenaline, by interfering with the Mg^{2+}-dependent vesicular storage mechanisms for amines. Thus, reserpine treatment leads to marked depression of adrenergic sympathetic neurotransmission and cardiovascular homeostasis, i.e., hypotension. In addition to this effect, relatively short term treatment with reserpine was recently reported to decrease the NPY-LI level in a tissue-dependent manner. Chlorisondamine, a ganglion blocking agent, blocks this tissue-specific decrease in NPY-LI seen with reserpine but not the decrease in noradrenaline, suggesting that this decrease in NPY-LI is dependent on a neurogenic mechanism unlike the decrease of catecholamines and that an increase in sympathetic nerve activity associated with reserpine treatment is essential for its effect in depleting sympathetic nerve endings and organs of NPY-LI (19, 25–27).

The effect of reserpine in causing tissue-specific decreases in the level of NPY could be due to increased degradation of NPY (possibly as a result of its increased release from nerve endings after increases in sympathetic nerve activity) or to tissue-specific decreases in the biosynthesis of NPY peptides, involving NPY gene transcription. The present results indicate that short term treatment with reserpine probably causes tissue-specific increases in NPY degradation, because treatment with reserpine for 1 day did not affect the NPY mRNA abundance in the adrenal gland (Figs. 1 and 6A).

Unexpectedly, we found that, after an initial decrease, the NPY level in the adrenal gland gradually increased with reserpine treatment. This increase was associated with prolonged increases in the abundance of mature NPY mRNA and putative NPY pre-mRNA species, which increased markedly and to similar extents (Figs. 4 and 5). These findings suggest that the increase seen with long term treatment (5 days) with reserpine was due to prolonged transcriptional activation of the NPY gene. Reserpine had a specific effect on expression of the neuron-specific NPY gene and did not affect that of the constitutive β-actin gene, implying that NPY gene expression is physiologically important for regulation of the blood pressure.

Results similar to our biochemical data were obtained by Schalling and collaborators (34, 35) in *in situ* hybridization studies. They reported that a single relatively high dose of reserpine (5 or 10 mg/kg) causes a rapid increase in the NPY mRNA level, mainly in most chromaffin cells in the adrenal medulla, within 3 days but they observed no initial decrease in peptide levels. The reason why they did not detect this initial decrease in NPY, observed in the present study and reported elsewhere (20, 23–26), may have been because it was masked by the increased biosynthesis of peptides following the rapid increase in NPY mRNA abundance induced by a high dose of reserpine. This masking is also the reason for the short duration of reserpine-induced decreases in NPY-LI in various sympathetic organs. Therefore, even though the steady state NPY level is unchanged, both the biosynthesis and turnover (including release) of NPY peptides might be dynamically increased in the peripheral sympathetic organs in reserpinized animals. The idea of an increase in turnover of NPY peptide with reserpine treatment is supported by the present finding that the increases in NPY peptide levels of the control (vehicle-treated stress) and reserpinized animals were relatively lower than those in the NPY mRNA abundances [e.g., 100 versus 178% and 202 versus 470%, respectively, on day 5 after the first injection (Figs. 1 and 6A)]. The increase in NPY gene expression may be an adaptation mechanism for conserving sympathetic neurotransmission after catecholamine depletion. Similar increases in NPY gene expression after reserpine treatment have been observed in sympathetic ganglia (37).

Reserpine treatment did not affect the level of NPY mRNA or NPY in regions of the brain (Figs. 4 and 6B and Table 2),

suggesting that it has no direct effect on central and peripheral neuronal cells that express the NPY gene. Reserpine is also known to increase the levels of ENK peptides, TH, and dopamine β-hydroxylase enzyme molecules in the adrenal gland (44–49). Although the increases in the levels of ENK peptides and TH enzyme molecules are both due to increased protein synthesis, reserpine treatment results in an increase in the level of TH mRNA but a decrease in that of proenkephalin A mRNA (ENK mRNA) (50–53). Therefore, the effect of reserpine on ENK biosynthesis was interpreted to be due to an increase in posttranslational processing of proenkephalins and/or to blockade of release of ENK peptides as a result of a direct action on the cells (49, 52, 54). In contrast, prolonged induction of production of TH enzyme molecules has been observed following increased neuronal activity (44–46). This prolonged induction of production of enzyme molecules results from a rise in the TH mRNA level, most probably due to an increase in TH gene transcription via transsynaptic control (50, 51).

To elucidate the involvement of nerve activity in the elevation of NPY gene expression (probably transcriptional activation) in peripheral sympathetic organs seen with reserpine, we transected the splanchnic nerve to eliminate the effect of increased sympathetic nerve activity on chromaffin cells (Fig. 8). We found that transection of the splanchnic nerve alone did not change the NPY mRNA level in the adrenal gland, indicating that under normal conditions the activity of the afferent splanchnic nerve has no tonic effect on NPY gene expression. However, splanchnic transection clearly inhibited the reserpine-induced increase in NPY gene expression, suggesting that this increased NPY gene expression depends on transsynaptic activation due to a reflex increase in activity of the afferent nerve, as in the case of expression of TH enzyme molecules.

The effects of denervation of the splanchnic nerve on the mRNA levels of transmitter-synthesizing enzymes and neuropeptides as cotransmitters have been investigated (45, 49, 55, 56). Denervation alone did not change the TH activity or the catecholamine level in the rat adrenal gland but completely blocked reserpine-induced TH activity and the concomitant increase in the TH mRNA level, which is probably due to an increase in TH gene transcription (45, 50, 51, 55). In contrast, proenkephalin A gene (ENK gene) expression in the adrenal gland is markedly increased by denervation alone (56). These observations suggested that under ordinary conditions splanchnic nerve activity depresses ENK gene expression and the level of ENK peptides in chromaffin cells by a transsynaptic mechanism, whereas, conversely, TH gene expression is not affected by splanchnic nerve activity under ordinary conditions but is induced by transsynaptic activation with reserpine.

Our data on the effects of splanchnic denervation on NPY gene expression in the adrenal gland were similar to those on its effects on TH gene expression.

TH gene expression can also be induced by stress, probably due to transsynaptic induction of the splanchnic nerve (50). It is noteworthy that the NPY mRNA abundance in the adrenal gland increased gradually in rats treated with the vehicle only and returned to the original level after the end of the injections (Fig. 6A), suggesting that stress induced by injections increases NPY gene expression in the adrenal gland, probably by the same transsynaptic induction mechanism. Insulin-induced hypoglycemic shock is also reported to increase NPY gene expression through a transsynaptic induction mechanism in the rat adrenal gland (33).

The effect of reserpine on NPY gene expression in the central nervous system is not observed, and the reason why NPY gene expression in the central neuronal cells fails to be induced by reserpine [relatively low dose (0.5 mg/kg)] is not known at present. However, if the action of reserpine is transsynaptically mediated, as in the adrenal gland, it is conceivable that the drug does not reflexively excite the central neuronal cells that express the NPY gene in the same manner. The idea might be supported by the report of Biguet *et al.* (51), indicating the absence of induction by reserpine (10 mg/kg) on TH gene expression in rat substantia nigra, in spite of the marked increase in TH mRNA abundance in rat adrenal gland and locus coeruleus, and also supported by the data that the late pronounced increase in NPY levels in brain regions following the initial decrease are observed after kainic acid-induced seizures in the rat (57).

For further investigation of the mechanism of transsynaptic induction of NPY gene expression in the adrenal gland, we used PC12 rat pheochromocytoma cells to examine the direct effects of reserpine, activation of nicotinic receptors, and membrane depolarization on NPY gene expression. We found that reserpine treatment reduced the NPY mRNA level in PC12 cells, rather than increasing it (Table 3). Reserpine did not change the amounts of ribosomal RNA (18 and 28 S) or of β-actin mRNA (data not shown). The similar decrease in mRNA abundance by reserpine was observed in the gene expression of proenkephalin A (ENK) in cultured chromaffin cells (52). Because at the same concentration (2.4 μg/ml) reserpine did not change the NPY mRNA abundance in NG108-15 hybrid cells (data not shown), the gene expression of both NPY and ENK might be suppressed by reserpine through the direct, cell-specific mechanism(s).

Nicotinic receptor activation produced significant but transient increases in the NPY mRNA abundance in PC12 cells. This suggested that transsynaptic induction of NPY gene expression in chromaffin cells was mediated by nicotinic acetylcholine receptors. The small transient effect of nicotinic activation might be due to rapid desensitization of nicotinic receptors. This putative rapid desensitization might explain the findings that nerve activity has no effects on NPY gene expression under ordinary conditions, whereas the increased splanchnic nerve activity with reserpine elevated the NPY gene expression in the adrenal gland.

To confirm the idea that nerve activation of chromaffin cells after nicotinic activation via transsynaptic control regulates NPY gene expression in the cells, we investigated the effect of persistent membrane depolarization (with 50 mM K^+ and 20 μM veratridine). These stimuli clearly increased NPY gene expression in PC12 cells. Because nicotinic receptor activation and membrane depolarization positively regulate NPY gene expression in PC12 rat pheochromocytoma cells, a tumor cell line of chromaffin cells, the increased NPY gene expression in chromaffin cells is probably induced by persistent membrane depolarization following repetitive nicotinic activation due to increased splanchnic nerve activity with reserpine.

The precise intracellular mechanism(s) involved in regulation of NPY gene expression in chromaffin cells is unknown. Probably, influx of extracellular Ca ion and consequent activation of protein kinase C participate in transcriptional induc-

tion of this gene, because tetrodotoxin, an inhibitor of voltage-sensitive Na channels, did not block the induction with high potassium medium (Table 4) and because we have already reported that elevation of intracellular Ca by the calcium ionophore A23187 increases the NPY mRNA abundance in PC12 cells and activation of protein kinase C markedly induces NPY gene transcription in combination with cAMP-dependent protein kinase (30, 36).

The effect of membrane depolarization stimuli was also observed in another neural cell line, NG108-15 cells (data not shown). Thus, positive regulation of NPY gene expression by membrane depolarization seems to be a generalized phenomenon in neural cells that express the NPY gene. This positive regulation of NPY mRNA level by membrane depolarization is also similar to that of TH mRNA level in rat sympathetic neurons, whereas ENK gene expression is, conversely, negatively regulated in the rat (58, 59).

Thus, the present study clearly shows that NPY gene expression is positively regulated by reserpine treatment, innervation, nicotinic activation, and membrane depolarization. It is interesting that these regulations of gene expression of a cotransmitter, NPY, are quite similar to those of TH gene expression.

The changes in splanchnic nerve activity associated with environmental stimuli and/or other factors, such as treatments with drugs, alter the steady state level of NPY mRNA, thereby altering synaptic neurotransmission (especially sympathetic tramsmission). Obviously, reserpine has a prolonged effect in inducing NPY gene expression in the adrenal gland of rats. Thus, this system is a good *in vivo* model for use in studies on the biochemical basis of storage of information for synaptic plasticity (60).

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