

Expression of Catecholamine-Synthesizing Enzymes, Peptidylglycine α -Amidating Monooxygenase, and Neuropeptide Y mRNA in the Rat Adrenal Medulla After Acute Systemic Nicotine

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Abstract

The expression of catecholamine-synthesizing enzymes in the adrenal medulla is upregulated in parallel by stress and pharmacological treatments. In this study we examined whether a neuropeptide and its processing enzyme are regulated in parallel with catecholamine enzyme genes after drug treatment. Because the main effect of stress on the adrenal medulla is via splanchnic nerve stimulation of nicotinic receptors, we used nicotine to stimulate the medulla and visualized expression of catecholamine enzyme genes, the medullary peptide neuropeptide Y (NPY), and the neuropeptide-processing enzyme peptidylglycine α -amidating monooxygenase (PAM) by *in situ* hybridization quantified by image analysis of autoradiographic images. Rats received a single injection of nicotine (0, 1, or 5 mg/kg sc). Six hours later, rats were transcardially perfused. Free-floating adrenal gland sections were hybridized with ³⁵S-labeled cDNA probes for tyrosine hydroxylase (TH), dopamine β -hydroxylase (DBH), phenylethanolamine N-methyltransferase (PNMT), PAM, and NPY. Nicotine treatment upregulated the expression of TH, PNMT, and NPY genes in a dose-dependent fashion. Small but nonsignificant increases were observed in DBH and PAM mRNA levels. These results suggest that common transcriptional activation mechanisms may upregulate both catecholamine and neuropeptide synthesis in the adrenal medulla after nicotinic stimulation.

Index Entries: Gene expression; cholinergic receptors; stress; neuropeptides; tyrosine hydroxylase; dopamine β -hydroxylase; phenyl-ethanolamine N-methyltransferase.

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Introduction

Environmental stress or pharmacological treatments which induce sympathetic responses activate the adrenal medulla by neuronal and nonneuronal mechanisms. Stress- or drug-induced secretion of medullary catecholamines and coreleased neuropeptides (Lundberg et al., 1985; Allen et al., 1986) is accompanied by altered gene expression, including increased expression of catecholamine-synthesizing enzymes (MacMahon et al., 1992; Wessel and Joh, 1992) and neuropeptides (Hiremagalur et al., 1994; Schalling et al., 1991) and decreased expression of some genes, such as the norepinephrine transporter (Cubells et al., 1995).

Neuronal activation of the adrenal medulla is largely mediated by the splanchnic nerve, which releases acetylcholine and neuropeptide cofactors onto nicotinic and muscarinic cholinergic receptors of the adrenal medulla (Ungar and Phillips, 1983). Systemic administration of nicotine emulates many of the pharmacological effects of stress by direct stimulation of adrenal nicotinic receptors (Fossom et al., 1991); it also affects nicotinic receptors in the central nervous system which results in indirect adrenal activation (Slotkin and Seidler, 1976; Fossom et al., 1991). Thus, nicotine causes adrenal catecholamine secretion (Slotkin and Seidler, 1976; Craviso et al., 1993), neuropeptide synthesis (e.g., calcitonin-gene related peptide [CGRP] and neuropeptide Y [NPY; Höfle et al., 1991]) and release (e.g., enkephalin, measured *ex vivo*; Eiden et al., 1984), and tyrosine hydroxylase enzyme activation (Fossom et al., 1991).

There have been several reports that systemic nicotine causes upregulation of catecholamine-synthesizing enzyme genes and neuropeptide genes, which parallels the induction of gene expression caused by stress or drugs such as reserpine (Stachowiak et al., 1990; Fossom et al., 1991; Höfle et al., 1991; Hiremagalur and Sabban, 1995). Previous studies have employed northern or dot blots to detect changes in mRNA levels. In this study, we employed quantitative *in situ* hybridization to detect upregulation of medullary gene expression. The expression of three catecholamine-synthesizing enzymes was examined: tyrosine hydroxylase (TH, EC 1.14.16.2), dopamine β -hydroxylase (DBH, EC 1.14.17.1), and phenylethanolamine N-methyltransferase (PNMT, EC

2.1.1.28). We also characterized changes in NPY mRNA as a representative neuropeptide colocalized with the adrenal catecholamines. For a neuropeptide-synthesizing enzyme gene, we examined the expression of peptidylglycine α -amidating monooxygenase mRNA (PAM, EC 1.14.17.3). Post-translational processing by PAM is essential for the full biological activity of many neuropeptides, including the medullary peptide NPY (Eipper et al., 1992).

Methods

Animals and Treatment

Adult male Sprague-Dawley rats (300–400 g) were group housed with *ad libitum* access to rodent chow and water. Rats received a single sc injection of either 0, 1, or 5 mg/kg nicotine (Sigma) in 1 mL/kg 0.15M NaCl ($n = 3$ –5/treatment). Six hours after treatment, rats were overdosed with sodium pentobarbital and transcardially perfused with 100 mL preperfusion solution (0.9% saline, 0.5% NaNO₂, and 10 U heparin/mL) to clear the blood, then perfused with 400 mL ice-cold 4% paraformaldehyde in 0.1M sodium phosphate buffer. The brains were removed, blocked, postfixed for 1–2 h in 4% formalin, then cryoprotected overnight in 30% sucrose.

In Situ Hybridization

Forty-micron sections were cut through the adrenal gland at -16°C on a sliding microtome. Alternate sections for *in situ* hybridization with the five cDNA probes described below were collected into 20 mL glass scintillation vials containing ice-cold 2X SSC (0.3M NaCl, 0.03M Na-Citrate). The SSC was pipetted off, and sections were suspended in 1 mL of prehybridization buffer (50% formamide, 10% dextran sulfate, 2X SSC, 1X Denhardt's solution, 50 mM DTT, and 0.5 mg/mL denatured salmon sperm DNA). After 1 h prehybridization at 48°C , labeled denatured cDNA probes (1×10^7 CPM) were added to the vials and hybridized overnight at 48°C . Following hybridization, the sections were extensively washed at 15-minute intervals in decreasing concentrations of SSC (2, 2, 1, 0.5, 0.25, 0.125, and

0.125X) at 48°C. The tissue sections were then mounted on gelatin-subbed slides, air-dried, and apposed to Kodak X-Omat autoradiographic film at 4°C. Exposure times were varied by probe (6–24 h for TH and NPY, and 48–96 h for DBH, PNMT, and PAM) in order to obtain autoradiographic images within a linear range of optical density. Some slides were subsequently dipped in undiluted Kodak NTB-2 photoemulsion and stored in light-tight boxes at 4°C for 1–4 wk. After development in Kodak D-19, the slides were counterstained with Cresyl violet and coverslipped.

Tissue sections from one rat from each treatment condition were hybridized within the same vial and apposed to film together on the same microscope slide. Sections from different rats were identified by punctures or nicks made in the adrenal gland during sectioning. Thus, *in situ* hybridization was carried out on representative members of each experimental group at the same time under identical conditions, allowing direct comparison of mRNA expression.

cDNA Probes

The cDNA probes for the catecholamine enzymes were cloned and sequenced in our laboratory: for TH, a 1.6-kb *KpnI-EcoRI* restriction fragment; for DBH, a 1.4-kb *EcoRI* fragment; for PNMT, an almost full-length *PstI* 1.0-kb fragment. The NPY cDNA probe was a 0.5-kb *EcoRI* restriction fragment comprising most of the cDNA of rat preproNPY (Higuchi et al., 1988; provided by S. Chua of Rockefeller University). The PAM cDNA probe was a 1.0-kb *EcoRI-BstEII* restriction fragment (Schafer et al., 1992; provided courtesy of R. Mains and B. Eipper of Johns Hopkins University).

Image Analysis

Relative optical density was measured for four adrenal tissue sections from each rat. Autoradiographic films were digitized with an MCD CCD72 monochrome video camera using a Research Imaging Systems Northern Light illuminator. With NIH Image (W. Rasband), the adrenal medullas were circled and the average pixel density calculated. The medullary optical density was averaged across four tissue sections per rat. Significant differences were detected by ANOVA and Fishers LSD post hoc test using StatView statistical software.

Results

The labeling of TH, DBH, PNMT, and PAM mRNA was homogenous throughout the adrenal medulla (Figs. 1, 2). A patchy distribution of NPY mRNA was observed after vehicle treatment, with low levels of expression punctuated by clumps of heavily labeled cells (Fig. 2).

Two responses in gene expression were observed in the adrenal medulla after systemic nicotine (Fig. 3). For TH, NPY, and to a lesser extent, PNMT, nicotine treatment induced an upregulation of mRNA levels. The increase in mRNA expression was dose-related for NPY. Nicotine treatment made the pattern of NPY expression more homogenous than in the control group. In contrast, DBH and PAM mRNA levels showed only small and nonsignificant increases in mRNA level 6 h after both 1 and 5 mg/kg nicotine.

Discussion

Of the catecholamine genes, expression of TH and PNMT were elevated 6 h after 1 or 5 mg/kg nicotine compared to vehicle treatment; DBH expression was not significantly elevated. The expression of the neuropeptide gene NPY was greatly elevated by nicotine, but expression of PAM, the final enzyme in neuropeptide processing, was not significantly increased. Using quantitative *in situ* hybridization, a dose-dependent effect of nicotine was observed on NPY mRNA levels. Thus, both the catecholamine-synthetic pathways and the neuropeptide-synthetic pathways are upregulated after a pharmacological treatment that stimulates catecholamine and neuropeptide secretion. These results are relevant for the genomic activation of the adrenal medulla during stress, when nicotinic cholinergic receptors are stimulated by acetylcholine release from the splanchnic nerve.

Our results are consistent with other observations of the acute effects of nicotine on gene expression *in vivo*. Fossom et al. showed that nicotine (1 mg/kg) increased TH enzyme activity and protein levels within 10 min (Fossom et al., 1991), by upregulating both TH mRNA synthesis and transcription rate (Fossom et al., 1991). Hiremagular et al. demonstrated by Northern blot that

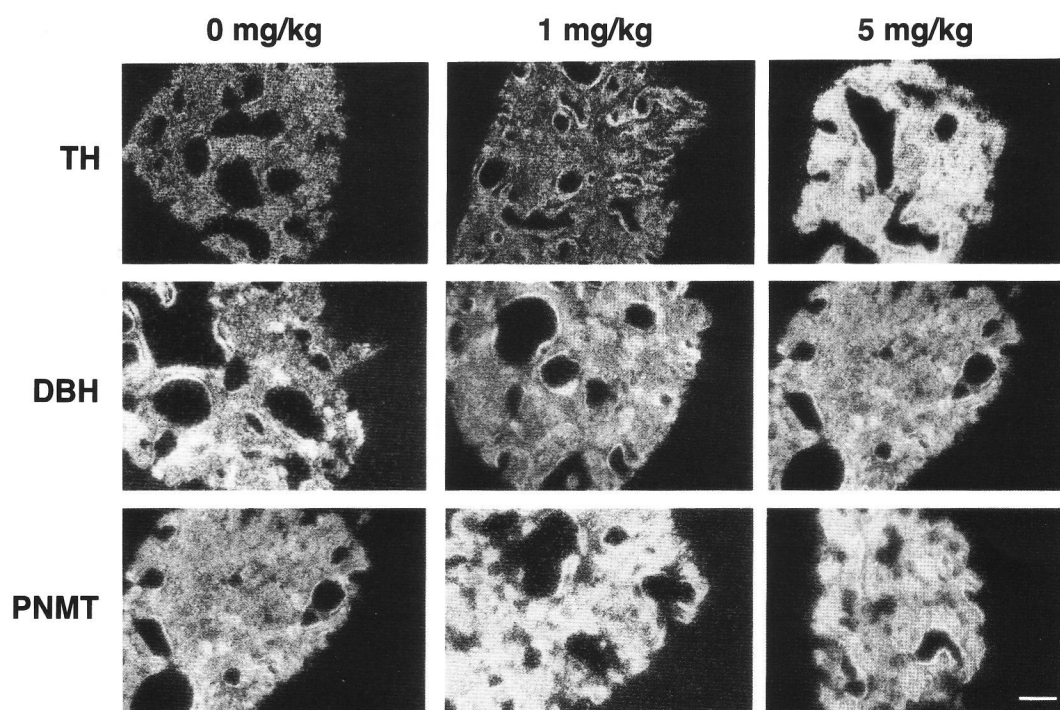


Fig. 1. Dark-field photomicrographs of *in situ* hybridization for TH, DBH, and PNMT in the adrenal gland from emulsion-dipped slides in vehicle- and nicotine-treated rats. An increase in TH mRNA (**top**) and PNMT mRNA (**bottom**) was observed in the adrenal medulla after 1 and 5 mg/kg nicotine. No change was observed in DBH mRNA levels (**middle**). Specific mRNA labeling was limited to the adrenal medulla for all three probes. Scale bar in lower right, 200 μ .

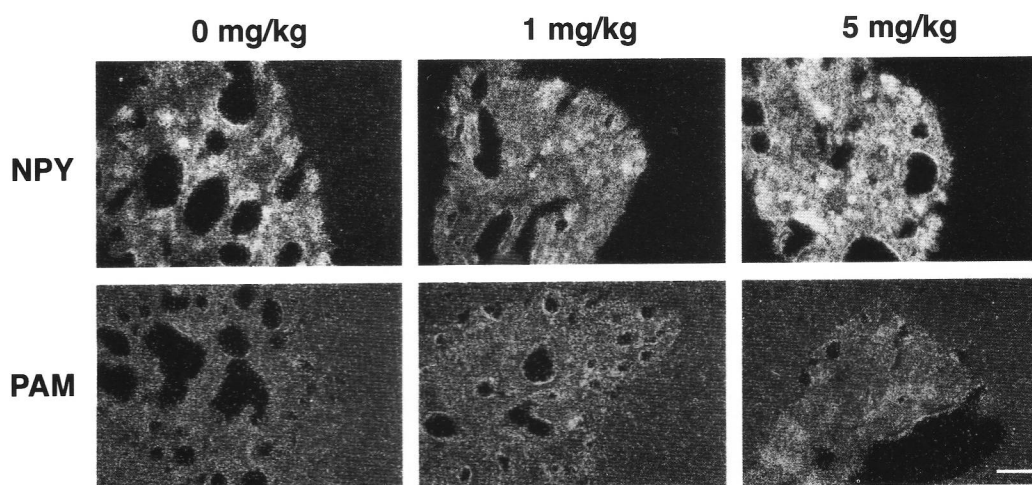


Fig. 2. Dark-field photomicrographs of *in situ* hybridization for NPY and PAM in the adrenal gland from emulsion-dipped slides in vehicle- and nicotine-treated rats. A significant increase in NPY mRNA (**top**) but not PAM mRNA (**bottom**) was observed in the adrenal medulla after nicotine treatment. Specific NPY mRNA labeling was limited to the adrenal medulla, but PAM mRNA was observed in both the adrenal medulla and cortex. Scale bar in lower right, 200 μ .

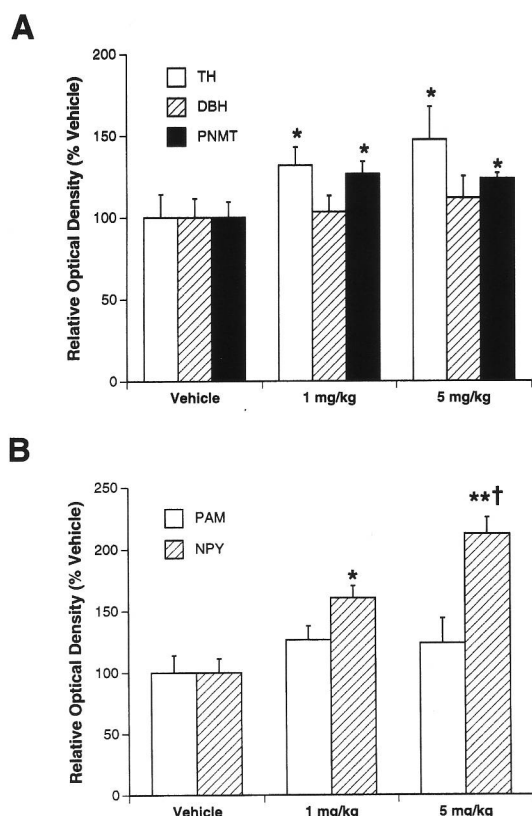


Fig. 3. (A) Induction of gene expression 6 h after acute nicotine treatment in the adrenal medulla measured as relative optical density of *in situ* hybridization autoradiograms. (A) Catecholamine-synthesizing enzyme mRNA levels. TH and PNMT, but not DBH, mRNA levels were significantly increased by nicotine ($*p < 0.05$ compared to vehicle-treated controls). (B) NPY and PAM mRNA levels. NPY, but not PAM, mRNA levels were significantly increased by nicotine treatment ($*p < 0.05$, $**p < 0.005$ compared to vehicle-treated controls; $†p < 0.05$ compared to 1 mg/kg nicotine).

acute nicotine treatment (5–15 mg/kg) caused upregulation of TH and NPY mRNA after 12 and 24 h (Hiremagalur and Sabban, 1995). Höfle et al. showed that nicotine (5 mg/kg) upregulates CGRP as well as NPY mRNA 24 h later (Höfle et al., 1991). Nicotine can increase PNMT gene expression in adrenal medulla chromaffin cells *ex vivo* (see Wan et al., 1991; Evinger et al., 1994). Our report demonstrates that upregulation of TH, PNMT, and NPY genes can be detected within 6 h of nicotine treatment *in vivo*.

Nicotine has also been reported to increase DBH gene expression but with a slower time course compared to TH and NPY (Höfle et al., 1991; Hiremagalur and Sabban, 1995); therefore we might have observed a significant increase in DBH and PAM gene expression if we had examined time-points later than 6 h. There are many parallels between PAM and DBH: Both are copper- and ascorbate-dependent mono-oxygenases anchored to the inner membrane of the vesicle, and they share 31% identity in amino acid sequence within their active sites (Stewart and Klinman, 1988; Eipper et al., 1992). The time-course of PAM induction might also parallel the time-course of DBH induction after nicotine treatment. For example, after reserpine treatment, PAM and DBH show increasing levels of expression that contrast with declining levels of TH and NPY expression at 24 and 48 h (Haupt et al., 1994). Thus, PAM and DBH, both vesicular enzymes, respond slowly to stimulation whereas TH and PNMT, both cytoplasmic enzymes, respond quickly. The reasons for the differential time-courses of activation are obscure.

Although the nicotinic receptor and its coupling to cellular activation are well characterized, the second messenger and transcription factors required for nicotine's effects on adrenal medulla gene expression are not completely known. Activation of protein kinase A (PKA) is probably required, because nicotine does not increase TH and DBH mRNA in adrenal-derived PC12 cell lines that are PKA-deficient (Hiremagalur et al., 1993).

The cAMP response element binding protein (CREB) and c-Fos are two PKA-regulated transcription factors that might control catecholamine and neuropeptide gene expression in the adrenal medulla. The TH promoter contains a CRE and an AP-1 binding site where CREB and c-Fos might upregulate TH expression (Kim et al., 1993). Nicotine increases phosphorylation of CREB *in vivo* (Hiremagalur and Sabban, 1995), which might upregulate transcription of neurotransmitter genes either directly or indirectly by inducing *c-fos* expression. Nicotine (Stachowiak et al., 1990; Koistinaho, 1991) and other stressful treatments (e.g., cold stress [Miner et al., 1992] or reserpine [Wessel and Joh 1992]) also induce *c-fos* gene expression in the adrenal medulla, but *c-fos* is expressed simultaneously and independently of

TH induction (Craviso et al., 1995). Therefore, *c-fos* and catecholamine gene expression may occur in parallel. Consistent with a role for CREB, but not *c-fos* in TH induction, the CRE site, but not the AP-1 site, of the TH promoter is required for nicotinic upregulation of transiently transfected TH promoter-CAT constructs in PC12 cells (Hiremagalur et al., 1993). Because the promoters of the DBH (Lamouroux et al., 1993) and NPY (Minth and Dixon, 1990) genes contain CRE or AP-1 elements, parallel mechanisms may regulate the other catecholamine-synthesizing genes and neuropeptide genes in the adrenal medulla, but they have not been characterized after nicotine treatment. The promoter of the PAM gene has not yet been isolated.

The PNMT promoter does not contain CRE or AP-1 elements, but it does contain a glucocorticoid-response element (Ross et al., 1990), and stress-induced increases in PNMT gene expression require adrenocortical activation (Viskupic et al., 1994). Accordingly, elevation of PNMT gene expression after nicotine treatment may depend on glucocorticoid secretion induced by nicotine treatment. The promoter of the PAM gene has not yet been isolated.

In summary, this *in situ* hybridization study demonstrated parallel upregulation of some neurotransmitter genes (TH, PNMT, and NPY) within 6 h of acute nicotine treatment, but showed that mRNA levels of other neurotransmitter-processing enzymes (DBH and PAM) were not significantly elevated. Because nicotinic receptors mediate, in part, the response of the adrenal medulla to stress, endogenous adrenal activation may induce similar patterns of gene transcription. The increased levels of catecholamine-synthesizing enzyme and neuropeptide gene expression may be important for maintaining the synthesis and secretion of catecholamines and peptides by the adrenal medulla after an acute stress.

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