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Differential In Vivo Regulation of mRNA Encoding the Norepinephrine Transporter and Tyrosine Hydroxylase in Rat Adrenal Medulla and Locus Ceruleus

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Abstract: To investigate the regulation of norepinephrine transporter mRNA in vivo, we analyzed the effects of reserpine on its expression in the rat adrenal medulla and locus ceruleus. First, PCR was used to clone a 0.5-kb rat cDNA fragment that exhibits 87% nucleotide identity to the corresponding human norepinephrine transporter cDNA sequence. In situ, the cDNA hybridizes specifically within norepinephrine-secreting cells, but in neither dopamine nor serotonin neurons, suggesting strongly it is a partial rat norepinephrine transporter cDNA. Reserpine, 10 mg/kg administered 24 h premortem, decreased steady-state levels of norepinephrine transporter mRNA in the adrenal medulla by ~65% and in the locus ceruleus by ~25%, as determined by quantitative in situ hybridization. Northern analysis confirmed the results of the in situ hybridization analysis in the adrenal medulla but did not detect the smaller changes observed in the locus ceruleus. Both analyses showed that reserpine increased tyrosine hydroxylase expression in the adrenal medulla and locus ceruleus. These results suggest that noradrenergic neurons and adrenal chromaffin cells can coordinate opposing changes in systems mediating catecholamine uptake and synthesis, to compensate for catecholamine depletion. **Key Words:** Reserpine—Neurotransmitter uptake—Northern blot—In situ hybridization—Catecholamines—Chromaffin cell—cDNA—Polymerase chain reaction.
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The synaptic action of norepinephrine (NE), like that of other monoamine transmitters, is inactivated largely by NaCl-dependent uptake into presynaptic noradrenergic neurons (Axelrod, 1971). Amara and colleagues (Pacholczyk et al., 1991) recently cloned the human cDNA encoding the NE transporter (NET) that mediates this uptake. The observation that NET cDNA is highly similar in sequence to that encoding the GABA transporter (Guastella et al., 1990) soon led to the cloning of cDNAs for many other sodium-dependent transporters, such as those for dopamine (Shimada et al., 1991; Usdin et al., 1991) and serotonin (Blakely et al., 1991; Hoffman et al., 1991), thus defining a new gene family (Uhl, 1992; Amara and Kuhar, 1993).

The foregoing advances set the stage for direct demonstrations that pharmacologic alteration of neurotransmission can change the in vivo expression of genes encoding neurotransmitter transporters. For example, measurements of mRNA from rat brain tissue samples showed that selective inhibitors of dopamine and serotonin uptake, respectively, diminish levels of mRNA encoding the dopamine transporter in substantia nigra (Xia et al., 1992) and the serotonin transporter in the raphe nuclei (Lesch et al., 1993). Conversely, in situ hybridization data suggest that short- or long-term administration of desipramine (an NE-uptake blocker) increases NET mRNA expression in the locus ceruleus (Szot et al., 1993).

In the present article, we characterize NET gene regulation in vivo by comparing the effects of reserpine on levels of the mRNAs encoding the NET and the rate-limiting enzyme for catecholamine biosynthesis, tyrosine hydroxylase (TH; EC 1.14.16.2), in rat adrenal medulla and locus ceruleus. We chose reserpine as a pharmacological tool because it exerts dramatic and well-characterized effects upon the expression of TH in both the adrenal medulla (Müeller et al., 1969; Joh et al., 1973; Faucon Biguet et al., 1986) and the locus

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Abbreviations used: DEPC, diethyl pyrocarbonate; NE, norepinephrine; NET, norepinephrine transporter; OD, optical density; SDS, sodium dodecyl sulfate; SSC, 150 m*M* NaCl/15 m*M* sodium citrate; TH, tyrosine hydroxylase (EC 1.14.16.2).

ceruleus (Reis et al., 1974, 1975; McMahon et al., 1990). In addition, the observation by Lee and colleagues (1983), that chronic reserpine diminishes NE uptake and [^{3}H]desipramine binding in rat cortex, suggested that reserpine might change expression of the mRNAs encoding TH and the NET in opposite directions. A preliminary report of the present work appeared recently (Cubells et al., 1993).

EXPERIMENTAL PROCEDURES

PCR cloning of rat NET cDNA

The template for the PCR reactions was prepared from PC-12 cell-derived poly(A)$^+$ RNA using avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim). PCR primers, synthesized by Bio-Synthesis, Inc. (Lewisville, TX, U.S.A.) corresponded to base pairs 990–1,017 and 1,489–1,512 of the human NET cDNA (Pacholczyk et al., 1991; sense: 5′-CAGTTACCAACAAATTTGACAACAACTGT-3′; antisense: 5′-GTCGTTTCGGAACCTGTCCACTCC-3′). PCR reactions, performed according to the manufacturer's protocol (Amplitaq Kit, Perkin-Elmer Cetus), contained 0.2 μg of each primer. Denaturation, annealing, and synthesis steps were run respectively at 94°C for 1 min, 54°C for 2 min, and 72°C for 3 min for a total of 35 cycles, except that the synthesis step lasted 5 min during the first and last cycles to ensure proper extension of DNA molecules. The 0.5-kb product of this reaction was then subcloned and sequenced by the dideoxynucleotide method.

Drug treatments

Male Sprague–Dawley rats (Charles River) and, in the case of one northern analysis experiment, male Fischer rats (Charles River), weighing between 200 and 300 g, were housed two to three per cage and given free access to food and water in a temperature-controlled room maintained on a 12-h light/dark cycle. All injections were given 3–5 h after "lights-on." Each rat received either a single subcutaneous injection of reserpine (Sigma Chemical) 10 mg/kg dissolved in 20% ascorbic acid solution or a 1 ml/kg dose of the ascorbic acid vehicle. This dose of reserpine has been previously shown to produce near-complete, long-lasting depletion of both central and peripheral stores of catecholamines (Schalling et al., 1991; Jenn-Tser et al., 1993). Rats were killed by decapitation (northern analysis experiments) or by overdose with pentobarbital (in situ hybridization experiments) 24 h after injection. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Cornell University Medical College.

Quantitative in situ hybridization

Rats were perfused transcardially with normal saline containing 10 U/ml heparin and 0.5% $NaNO_2$, followed by 4% formaldehyde in 0.1 *M* phosphate buffer. Blocked brains, adrenal glands, and, in some cases, superior cervical ganglia, were postfixed in cold 4% formaldehyde for 1 h, and then stored in 0.1 *M* phosphate buffer prepared in diethyl pyrocarbonate–treated water (DEPC-H_2O; Sambrook et al., 1989). Before sectioning, tissue was cryoprotected overnight in 30% sucrose prepared in DEPC-H_2O; control tissue was marked by notching (adrenal glands) or needle puncture (brain blocks). Frozen sections (40 μm) were cut on a sliding microtome and placed into 2× SSC (1× SSC = 0.15 *M* NaCl/0.015 *M* sodium citrate) prepared in DEPC-H_2O. To ensure that control and reserpine-treated samples were subject to identical hybridization conditions, sections from one control and one reserpine-treated rat were collected and processed for in situ hybridization in one vial for each probe. The hybridization conditions have been described previously (Wessel and Joh, 1992).

All in situ hybridization experiments used ^{35}S-labeled cDNA probes prepared by the random primer method. The rat NET probe is described here, and the 1.6-kb rat TH probe has been used extensively in previous work from this laboratory (Kim et al., 1993). Probe-labeling efficiency varied from 2.0 to 4.0 × 10^6 dpm/ng; the final hybridization buffers contained 2 × 10^7 dpm/ml.

Autoradiograms of the tissue sections were prepared by exposure of Kodak X-Omat film to the ^{35}S-labeled sections at room temperature. Durations of film exposure were adjusted to ensure that the optical density (OD) readings for each tissue/probe combination were in the linear range. Exposures for TH-labeled samples ranged from 4 to 24 h, with adrenal medulla samples requiring less time than locus ceruleus samples. Exposures for NET-labeled sections ranged from 16 h to 4 days, with locus ceruleus samples requiring less time than adrenal medulla samples. For quantification, the x-ray film images of matched sections of adrenal medulla and locus ceruleus, from control and reserpine-treated rats, were analyzed on a Zeiss Ibas 20 image analysis system by one of the authors, who was blind to treatment condition. Standard curves, generated using Kodak neutral-density gelatin filters (step tablet; 404ST167), were used to determine mean OD per pixel on a gray scale ranging from 1 to 255, according to previously published procedures (Saji et al., 1994; Weiser et al., 1993, 1994). Background signal over a comparable area of tissue, from adrenal cortex for the adrenal medulla and ventral pons for the locus ceruleus, was subtracted from the total signal to generate specific OD for each sample. Mean specific OD for each rat was calculated for each probe within each anatomical region before statistical analysis. Sections from each reserpine-treated rat, along with paired vehicle-control sections, were always processed in one vial. OD measurements were compared in paired-sample *t* tests.

Northern blot analysis

Adrenal glands for northern analysis were dissected rapidly and frozen in liquid N_2. Sections of dorsal pons containing the locus ceruleus bilaterally were dissected by first carefully removing the cerebellum from the brainstem and then making coronal cuts at the pontine–midbrain junction and the caudal limit of the fourth ventricle. The resulting section was laid flat on its anterior surface, and the ventral half removed by a horizontal cut through the medial longitudinal fasciculus. The resulting dorsal pons section was then frozen in liquid N_2. Tissue samples were pooled from three to five rats per treatment group. Equal numbers of rats per group were used in each replication.

Poly(A)$^+$ RNA was extracted from cold-pulverized, proteinase K–digested tissue by oligo(dT) cellulose chromatography (Badley et al., 1988); 2–4 μg per lane of poly(A)$^+$ RNA was then size-separated by agarose gel electrophoresis (1% agarose, 6% formaldehyde) and transferred to Hybond-N membrane (Amersham). After baking under vacuum for 2 h, blots were hybridized to ^{32}P-labeled cDNA probes for rat NET, rat TH (Kim et al., 1993), and human α-tubulin

YRDALLTST(S)INCV(I)TSFI(V)SGFAIFSILGYMAHEHKVK(N)IEDV
ATEGAGLVFV(I)LYPEAISTLSGSTFWAVL(V)FFL(V)MLLALGLDS
SMGGMEAVITGLADDFQVLKRHRKLFTC(F)A(G)VTL(F)G(S)TFLLA
M(L)FCITKGGIYVLTLLDTFAAGTSILFAVLMEAIGVSWFY

FIG. 1. The amino acid sequence predicted by presumptive rat NET cDNA is very similar to that of the corresponding human NET sequence. A cDNA corresponding to base pairs 990–1,512 of the human NET gene (Pacholczyk et al., 1991) was cloned and sequenced as described in the text and its amino acid sequence deduced assuming the same reading frame as for the human NET cDNA. Only the residues in boldfaced type differed from those of the human sequence (shown in parentheses).

(Carroll et al., 1991), as follows. Prehybridization was performed for 45 min at 42°C in 9 ml of 50% deionized formamide, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 1 *M* NaCl. One to 2 ng of probe ($2.0–4.0 \times 10^6$ dpm/ng), in 1.2 ml of prehybridization solution containing 1 mg of sonicated salmon sperm DNA, was incubated at 100°C for 10 min, chilled on ice, and added to the unlabeled prehybridization solution. Hybridization at 42°C was then continued overnight (minimum of 8 h). Blots were washed at room temperature twice for 5 min in 2× SSC and once for 30 min in 0.1× SSC + 0.1% SDS before exposure to x-ray film. Two separate northern blot experiments were quantified by independent methods. In one set of experiments, x-ray film autoradiograms were scanned into digital memory and the OD of each band determined. In a separate experiment, northern blots were quantitated directly in a Molecular Dynamics Phosphorimager using Imagequant software.

Image processing

Sections processed for in situ hybridization and subsequently dipped in Kodak NTB2 photographic emulsion were photographed using Kodak tungsten-filament color slide film. The slides were scanned into digital memory using a Nikon LS-3510 AF film scanner attached to a Macintosh IIfx microcomputer, converted to black and white, and arranged in composites using Photoshop version 2.5 software (Adobe, Inc.). Figures prepared from x-ray film autoradiograms (see Figs. 2 and 6) were scanned directly into digital memory. In no case was any digital manipulation performed that altered the data.

RESULTS

Using PCR primers based on the published sequence of the human NET cDNA, and template prepared from PC-12 cell poly(A)$^+$ RNA, we isolated a 0.5-kb cDNA fragment corresponding to base pairs 990–1,512 of the human NET gene (Pacholczyk et al., 1991). The sequence (GenBank accession number L29573) is 87% identical to the nucleotide sequence of the corresponding region of the human NET cDNA. As shown in Fig. 1, the predicted amino acid sequence is 92% identical to the corresponding human peptide.

To confirm that the above cDNA would specifically identify NET mRNA, we examined its in situ hybridiza-

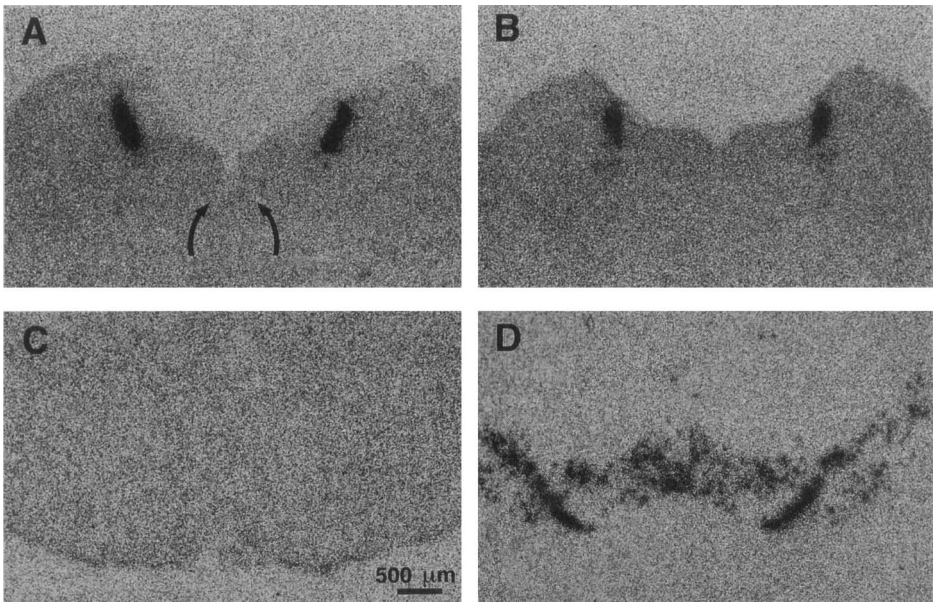

FIG. 2. The presumptive rat NET cDNA exhibits specific hybridization in situ to noradrenergic neurons, demonstrated by comparison with a TH probe. X-ray film autoradiograms of pontine (A and B) and ventral midbrain (C and D) sections show hybridization of the NET cDNA (A and C) compared with that of a rat TH cDNA (B and D). **A** and **B**: The NET probe hybridizes robustly in the locus ceruleus (A) in a pattern identical to TH hybridization (B). The arrows in A delineate the median raphe, which contains serotonergic neurons easily visualized with appropriate probes using identical in situ hybridization procedures (Wessel and Joh, 1992), but which are not labeled by the NET cDNA. **C** and **D**: In the midbrain, the NET probe (C) does not hybridize to dopaminergic neurons of the substantia nigra pars compacta and ventral tegmental area. These neurons are clearly labeled by the TH probe (D).

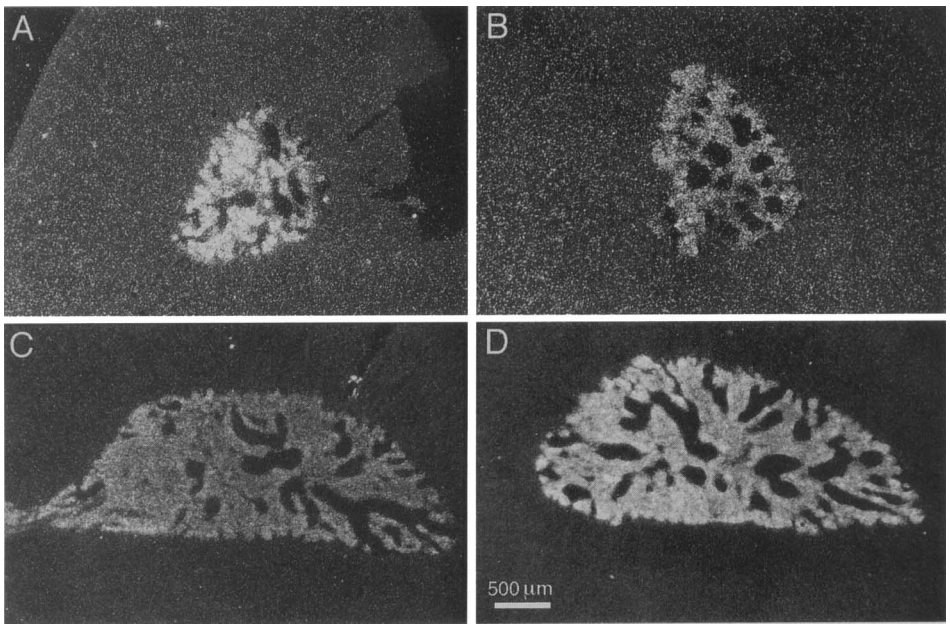

FIG. 3. The effects of reserpine on NET and TH gene expression in the adrenal medulla, visualized by in situ hybridization. After in situ hybridization as described in the text, sections (40 μm) were mounted on slides, dipped in photoemulsion, developed, and examined under dark-field optics. In adrenal sections hybridized to the NET probe (A and B) or a TH probe (C and D), specific hybridization occurs only in the medulla. Each pair of sections was processed in the same vial to ensure identical hybridization conditions; control sections were identified by notching them with a razor blade. **A** and **B**: Sections from a vehicle-injected control rat (A) and one given 10 mg/kg reserpine 24 h before perfusion (B) show clear diminution of NET message levels after reserpine. **C** and **D**: Sections from a vehicle-injected control rat (C) and one given 10 mg/kg reserpine 24 h before perfusion (D) show the robust increase in TH mRNA reported in many previous studies (see text).

tion to sections through several brain regions and through the adrenal gland and superior cervical ganglion. Figure 2 shows representative results from the brain, compared with in situ hybridization of a rat TH probe. The NET cDNA hybridizes robustly to neurons in the locus ceruleus (Fig. 2A), in a pattern identical to that of the TH probe (Fig. 2B), but not to serotonergic neurons of the median raphe (Fig. 2A, arrows). In the ventral midbrain, at the level of the substantia nigra pars compacta, the NET probe (Fig. 2C) did not hybridize to dopamine neurons easily visualized with the TH probe (Fig. 2D). In the periphery, the NET cDNA hybridized to chromaffin cells in the adrenal medulla but not to cells in the adrenal cortex (Fig. 3A). Scattered small cells within the adrenal medulla, probably representing sympathetic ganglion cells (Dagerlind et al., 1990), exhibited intense hybridization to the NET probe, as did cell bodies in the superior cervical ganglion (not shown). In summary, the NET cDNA specifically hybridized to noradrenergic cells but not to dopaminergic or serotonergic neurons.

To assess the regulation of NET mRNA, we subjected tissue sections from reserpine- and vehicle-treated rats to quantitative in situ hybridization. Figure 3 shows NET mRNA in the adrenal medulla of a rat given 10 mg/kg reserpine 24 h before perfusion (Fig. 3B) compared with that from a rat given ascorbic acid vehicle (Fig. 3A). NET hybridization signal is reduced dramatically in the reserpine-treated adrenal medulla compared with the control. In the locus ceruleus, reserpine elicited less dramatic changes (Fig. 4A and B). Reserpine increased the expression of TH in both the adrenal medulla (Fig. 3C and D) and locus ceruleus (Fig. 4C and D).

The results of quantitative analysis of the in situ hybridization experiments are shown in Fig. 5. In the adrenal medulla, NET optical densities were ~65% lower than those from control rats ($p < 0.005$), whereas in the locus ceruleus, they were ~25% lower than in controls ($p < 0.05$). In agreement with many previous studies from this laboratory and others (Mallet et al., 1983; Faucon Biguet et al., 1986; Wessel and Joh, 1992), reserpine increased levels of TH mRNA in both the adrenal medulla and locus ceruleus ($p < 0.01$).

In northern blots, the NET identified a major band of the same size (~5.8 kb; Pacholczyk et al., 1991; Ramachandran et al., 1993) as the rat neuronal NET mRNA (size data not shown). Relative changes in band intensities determined by measuring film ODs (Fig. 6) were comparable with those from blots prepared in a separate experiment, in which the radioactivity of bands was measured directly using a Molecular Dynamics Phosphorimager (data not shown). In either

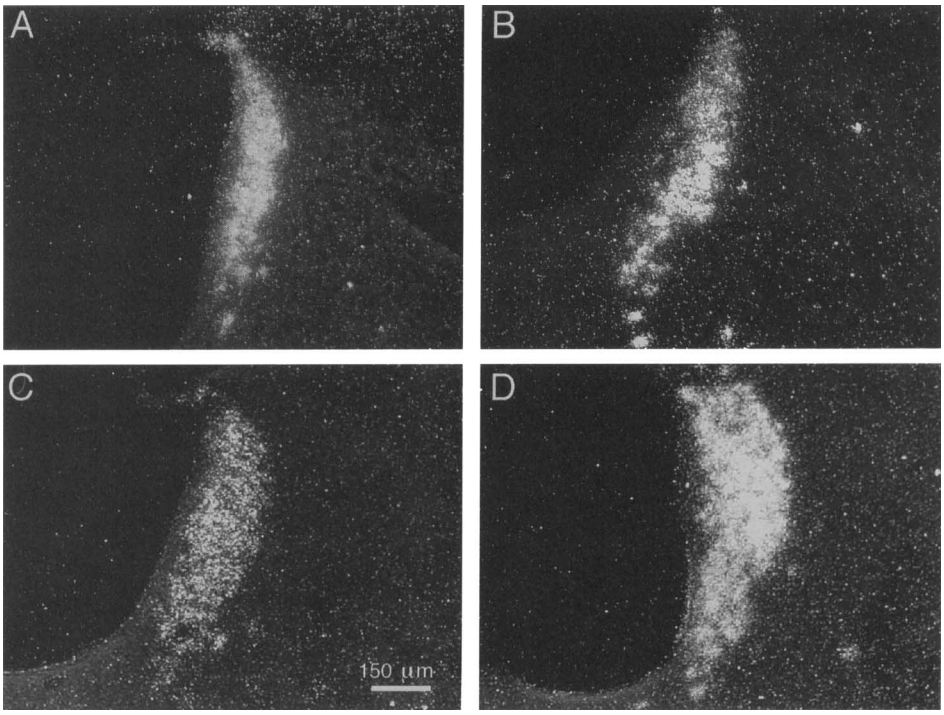

FIG. 4. The effects of reserpine on NET and TH gene expression in the locus ceruleus, visualized by in situ hybridization. After in situ hybridization as described in the text, sections (40 μm) were mounted on slides, dipped in photoemulsion, developed, and examined under dark-field optics. The NET (A and B) and TH probes (C and D) hybridized specifically in the locus ceruleus. Each pair of sections was processed in the same vial to ensure identical hybridization conditions; control sections were identified by needle puncture. **A** and **B**: Compared with the control locus ceruleus (A), NET mRNA in the reserpine-treated locus ceruleus (B) appeared mildly diminished. **C** and **D**: Hybridization of the TH probe was elevated in the locus ceruleus from rats given reserpine (D) compared with control (C).

case, northern data confirmed the results of the in situ hybridization experiments in the adrenal gland, as well as the effect of reserpine on TH mRNA in the locus ceruleus–containing samples of the dorsal pons. However, northern blot analysis did not detect the small reserpine-induced change in NET expression in the locus ceruleus evident with in situ hybridization.

DISCUSSION

This article describes a new rat NET cDNA isolated from PC-12 cells by using PCR. Two other cDNA fragments, that also appear to be partial rat NET cDNAs, have been isolated previously from PC-12 cells by similar methods (Ramachandran et al., 1993; Lorang et al., 1994). The cDNA described in this report corresponds to a region of the human NET cDNA just 3′ to the regions represented by the previously described rat NET cDNAs. All three rat cDNAs exhibit high degrees of sequence similarity to the corresponding regions of the human NET cDNA (Pacholczyk et al., 1991). The NET cDNA described in the present report hybridizes in situ to the noradrenergic neurons of the locus ceruleus but not to dopaminergic neurons or serotonergic neurons. In northern blots, our NET cDNA identifies an mRNA of a size identical to that encoding the rat neuronal NET (Pacholczyk et al., 1991; Ramachandran et al., 1993). Together, the foregoing data provide strong evidence that the cDNA described here is a partial rat NET cDNA.

Our results demonstrate that reserpine diminishes NET expression at the same time it increases TH expression. Abundant evidence indicates that, in the adrenal medulla, TH activity and expression rise in response to increased activity of the splanchnic nerve after reserpine administration or other stressful stimuli (Thoenen et al., 1969; Guidotti and Costa, 1977; Zigmond, 1979; Rius et al., 1994). In contrast, acetylcholine-stimulated release of catecholamines from isolated chromaffin cells is accompanied by diminished catecholamine uptake (Role and Perlman, 1983). NE transport and TH activity thus appear to play opposing roles in adrenomedullary catecholamine secretion (Perlman and Role, 1985). Up-regulation of TH expression after reserpine has been interpreted as a homeostatic mechanism that increases the catecholamine synthetic capacity of adrenal chromaffin cells in response to the reflexive increase in splanchnic nerve activity resulting from hypotension produced by reserpine-induced catecholamine depletion (Müeller et al., 1969; Thoenen et al., 1969; Joh et al., 1973). Decreased expression of the NET in adrenal medulla after

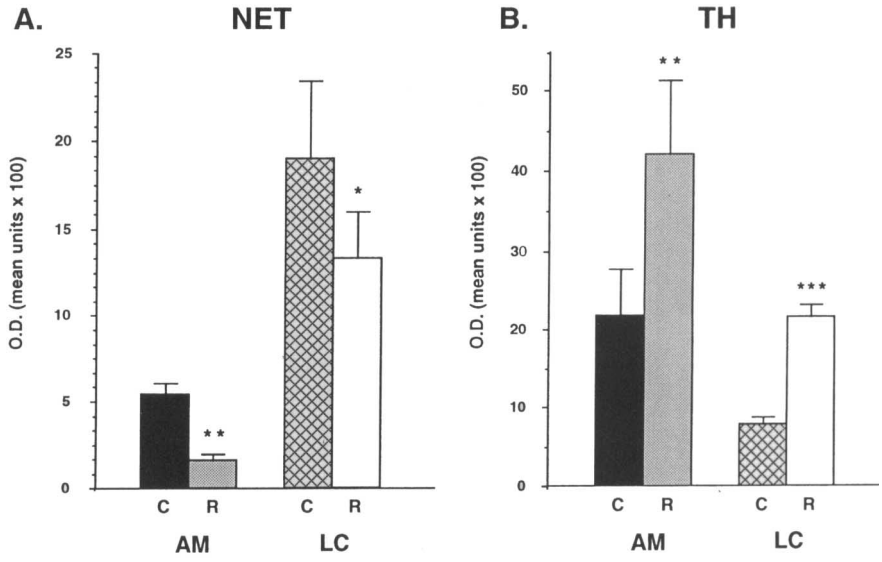

FIG. 5. The effects of reserpine on NET and TH mRNA in the adrenal medulla and locus ceruleus, determined by quantitative in situ hybridization. After in situ hybridization, x-ray film autoradiographic images were subjected to quantitative analysis. For each probe, OD within each adrenal medulla (AM) and locus ceruleus (LC) image was determined as described in the text, and a mean value for each rat calculated (n = 5 rats per group). Paired-sample *t* tests were used to compare results for each probe, within each tissue, for the reasons described in the text. Data are coded as raw OD values multiplied by 100 and presented as means ± SEM for control (C) and reserpine-treated (R) sections. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

reserpine treatment could also represent a homeostatic response of noradrenergic cells to increased demand for catecholamine secretion because it would presumably decrease the reuptake of NE and thereby enhance noradrenergic transmission. The differential effect of reserpine on TH and NET mRNA levels may therefore reflect coordination of *opposite* changes in TH and NET gene expression to produce a functionally *additive* response. The mechanisms responsible for this apparently coordinated regulation of the two genes remain to be determined.

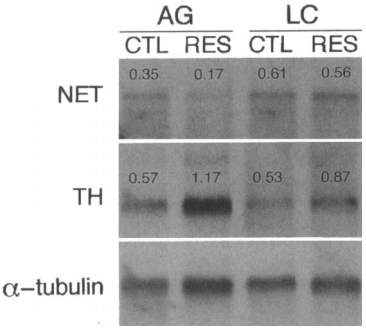

FIG. 6. The effect of reserpine on NET, TH, and α-tubulin mRNA expression in adrenal medulla and locus ceruleus, as determined by northern analysis. Poly(A)$^+$ RNA, extracted from whole adrenal glands and locus ceruleus–containing blocks of dorsal pons from four rats per group (CTL, 1 ml/kg ascorbic acid vehicle; RES, reserpine 10 mg/kg; killed 24 h after injection), was subjected to northern analysis. Blots were probed sequentially with cDNA probes for NET, TH, and α-tubulin (approximate band positions: 5.8, 2.1, and 1.8 kb, respectively). Autoradiograms were quantified by densitometry. Values shown for the NET and TH bands are relative OD, normalized to the corresponding α-tubulin OD. In a separate experiment (not shown) relative changes in NET message levels, determined by direct measurement of blot radioactivity in a Molecular Dynamics Phosphorimager, were comparable with those estimated by densitometry.

In the present experiments, northern blot analysis did not detect the 25% decrease in NET mRNA apparent by quantitative in situ hybridization in the locus ceruleus. Analysis of anatomically precise samples from individual rats by quantitative in situ hybridization is probably more sensitive to small changes in mRNA levels than northern analysis of mRNA pooled from multiple samples of dissected dorsal pons. Thus, the variability in tissue dissection, combined with relatively low yields of poly(A)$^+$ RNA obtained from the locus ceruleus–containing brain tissue, most likely diminished the sensitivity of the northern analyses sufficiently to obscure small changes in NET expression. Consistent with this interpretation, northern blotting detected a smaller magnitude increase in TH mRNA in the locus ceruleus–containing dorsal pons samples than did quantitative in situ hybridization in the locus ceruleus.

It is not clear why reserpine induces proportionately smaller changes in NET mRNA within the locus ceruleus than in the adrenal medulla under the present experimental conditions. Reserpine-induced changes in the expression of other catecholamine genes in the adrenal medulla and locus ceruleus usually parallel each other (Faucon Biguet et al., 1986; Richard et al., 1989). One factor that is potentially important in explaining the relative disparity in the present results is that baseline NET expression is substantially more robust in the locus ceruleus than in the adrenal medulla. It is thus possible that the molecular mechanisms supporting higher basal expression of NET mRNA in the locus ceruleus are less sensitive to attenuation after reserpine. Alternatively, if adrenal corticosteroids regulate NET expression, as suggested by Hirano and Kobayashi (1978), such a mechanism could explain the differential magnitude of the reserpine effect in the adrenal medulla. Reserpine elevates adrenal corticoste-

roid secretion (Lowy et al., 1990). Because the adrenal medulla receives most of its blood supply from the sinusoids of the adrenal cortex (Vinson and Hinson, 1992), it is exposed to much higher concentrations of corticosteroids than the locus ceruleus. Thus, suppression of NET expression by corticosteroids is one possible mechanism that could explain the differential effect of reserpine on NET mRNA in the adrenal medulla and locus ceruleus. Although such a mechanism is speculative at this point, it represents a plausible and experimentally testable hypothesis.

In summary, using a new cDNA probe to measure NET mRNA, we observed that 10 mg/kg reserpine, given 24 h premortem, decreased NET expression by ~65% in the adrenal medulla but only by 25% in the locus ceruleus. In either tissue, the reserpine-induced change in NET mRNA was opposite to that of TH mRNA, suggesting that noradrenergic neurons and adrenal chromaffin cells can coordinate opposing changes in systems mediating catecholamine uptake and synthesis, to compensate for catecholamine depletion.

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