

## Innervation-independent changes in the mRNAs encoding tyrosine hydroxylase and the norepinephrine transporter in rat adrenal medulla after high-dose reserpine

Joseph F. Cubells<sup>a,c</sup>, Harriet Baker<sup>a</sup>, Bruce T. Volpe<sup>b</sup>, Gerard P. Smith<sup>c</sup>, Sonal S. Das<sup>a</sup>, Tong H. Joh<sup>a,\*</sup>

<sup>a</sup>Laboratory of Molecular Neurobiology, Burke Medical Research Institute, 785 Mamaroneck Avenue, White Plains, NY 10605, USA

<sup>b</sup>Laboratory of Behavioral Neurology, Burke Medical Research Institute, 785 Mamaroneck Avenue, White Plains, NY 10605, USA

<sup>c</sup>E.W. Bourne Behavioral Laboratory, Department of Psychiatry, Cornell University Medical College, 21 Bloomingdale Road, White Plains, NY 10605, USA

Received 3 March 1995; revised version received 12 May 1995; accepted 19 May 1995

### Abstract

To determine whether a trans-synaptic mechanism triggered the effects of reserpine on adrenomedullary mRNAs encoding the norepinephrine transporter and tyrosine hydroxylase, we administered 10 mg/kg reserpine to rats after unilateral splanchnicotomy, and examined their adrenal medullas using quantitative *in situ* hybridization. Splanchnicotomy did not alter the decrease in norepinephrine transporter mRNA that follows reserpine administration, but diminished the reserpine-induced increase in tyrosine hydroxylase mRNA by almost 80%. Despite the latter effect, reserpine still induced a significant increase in tyrosine hydroxylase mRNA in denervated adrenal medullas, compared to vehicle-treated adrenal medullas. These results show that a trans-synaptic mechanism does not trigger the decrease in adrenomedullary norepinephrine transporter mRNA following reserpine. In addition, an innervation-independent mechanism mediates a portion of the reserpine-induced increase in adrenomedullary tyrosine hydroxylase mRNA.

**Keywords:** Adrenal chromaffin cells; Trans-synaptic gene induction; Catecholamine; Neurotransmitter uptake; *In situ* hybridization; Splanchnic nerve

The norepinephrine transporter (NET) catalyzes the NaCl-dependent uptake of norepinephrine into noradrenergic cells, and thereby terminates the synaptic action of norepinephrine. It is important to understand how the expression of mRNA encoding the NET is regulated by catecholamine-secreting cells, because this transporter plays such a central role in regulating noradrenergic transmission. Recent experiments demonstrated that administration of reserpine, an alkaloid that depletes cellular stores of catecholamines, substantially diminished the expression of NET mRNA in the adrenal medulla within 24 h of injection, while producing significant, but smaller, decreases in the locus ceruleus [2]. Consistent with results from numerous laboratories [4,10,19], reserpine also increased levels of mRNA encoding tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine bio-

synthesis, in both the adrenal medulla and locus ceruleus [2]. Reserpine thus produced opposing changes in the expression of NET and TH by catecholamine-secreting cells, *in vivo*. We hypothesized that these opposite absolute changes in NET and TH expression might be additive functionally, because diminished uptake and increased synthesis of norepinephrine both would be expected to enhance noradrenergic transmission. Indeed, coordination between enhanced release and diminished uptake of catecholamines by cultured adrenal chromaffin cells has been reported [14,15]. If such functional coordination of gene expression occurs within catecholamine-secreting cells, the simplest mechanism for producing it would be a common signal that triggers the decrease in levels of NET mRNA and the increase in TH mRNA.

Induction by reserpine of TH enzyme activity in the adrenal medulla [12], and of TH mRNA in the superior cervical ganglion [1] depends on the presence of intact preganglionic innervation to those structures [1,17]. The

\* Corresponding author. Tel.: +1 914 948 0050, ext. 2152; Fax: +1 914 948 9541.

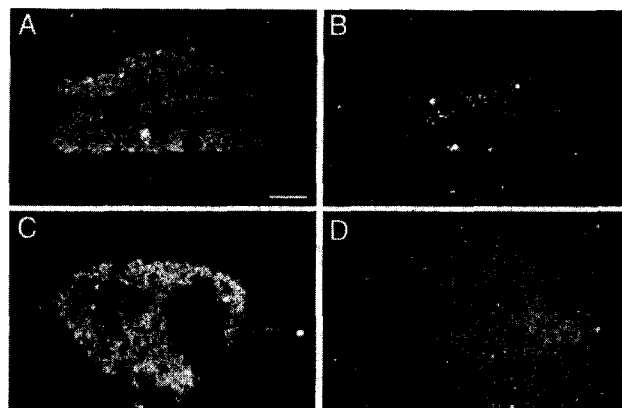


Fig. 1. The effects of reserpine on mRNA encoding the NET in intact and splanchnicotomized adrenal medulla. After in situ hybridization using an  $^{35}\text{S}$ -labeled probe for rat NET mRNA, sections were mounted on slides and dipped into Kodak NTB2 photographic emulsion, developed, counterstained with cresyl violet and photographed under dark-field optics using Kodak tungsten-filament color slide film. The resulting 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.). Micrographs representing each experimental condition are shown: (A) Intact-vehicle (1 ml/kg 20% ascorbic acid, s.c., 24 h prior to perfusion); (B) intact-reserpine (10 mg/kg s.c., 24 h prior to perfusion); (C) splanchnicotomy-vehicle; (D) splanchnicotomy-reserpine. Scale bar = 100  $\mu\text{m}$ .

classical paradigm for demonstrating this trans-synaptic mechanism of gene induction is to denervate the adrenal medulla or superior cervical ganglion unilaterally, and compare the effects of reserpine on gene expression in the intact and denervated structures [1,17]. Denervation of the adrenal medulla is easily accomplished by surgical transection of the splanchnic nerve [17]. To determine whether the effects of reserpine on mRNA encoding the NET and TH occur by a common, trans-synaptic, mechanism in the AM, we used in situ hybridization to compare levels of mRNA from the adrenal medullas of reserpine- and vehicle-treated rats that had undergone unilateral splanchnicotomy.

All animal procedures were approved by the Institutional Animal Use and Care Committee of the Cornell University Medical College. Male Sprague–Dawley rats, housed 2–3 per cage with free access to food and water under a 12 h light/12 h dark cycle, and weighing between 250–300 g at the time of surgery, underwent unilateral (L-sided) splanchnicotomy as follows. Under surgical anesthesia produced by a mixture of chloral hydrate and pentobarbital (Chloropent, 3 ml/kg, i.p.), a midline incision was made and the left adrenal gland and the area dorso-medial to it were identified. The left splanchnic nerve was dissected free from the surrounding tissue using the landmarks described by Lambert [8]. Two 3–0 silk sutures were tied tightly on the nerve about 1 cm apart and the nerve was cut between the sutures. Care was taken to avoid handling the adrenal gland and disturbing

its blood supply during the procedure. Examination at necropsy under a dissecting microscope confirmed that splanchnicotomies were complete, and that no regeneration had occurred.

After recovering from surgery for 1–3 weeks, rats were grouped into pairs in which one received a single injection of reserpine, 10 mg/kg s.c., and the other, 1 ml/kg s.c. of the 20% ascorbic acid vehicle. Rats were perfused 24 h after injection with heparinized 0.9% NaCl solution containing 0.05%  $\text{NaNO}_2$  followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Adrenal glands were then dissected, post-fixed for 1 h, and cryoprotected overnight in 30% sucrose prepared in diethylpyrocarbonate-treated  $\text{H}_2\text{O}$ . Prior to sectioning, adrenal glands were marked by needle puncture or razor nicks in the cortex, to identify the four treatment groups: intact-vehicle, splanchnicotomy-vehicle, intact-reserpine, and splanchnicotomy-reserpine. Sections 40  $\mu\text{m}$  thick were cut on a sliding microtome. To ensure identical hybridization conditions across groups, all sections from each pair of rats were processed for in situ hybridization in a single vial, using  $^{35}\text{S}$ -labeled cDNA probes for rat NET and TH precisely as described previously [2]. Hybridization signal on X-ray film autoradiograms was quantified by densitometry as described before [2]. In sections from one pair of rats, the radiolabeled NET probe degraded and failed to hybridize properly, so these sections were excluded from analysis. We thus analyzed data from 6 pairs of rats for TH mRNA, and from 5 pairs for NET mRNA.

The effect of reserpine on levels of NET mRNA in intact adrenal medullas was identical to that previously observed in rats that had not undergone surgery [2]. In re-

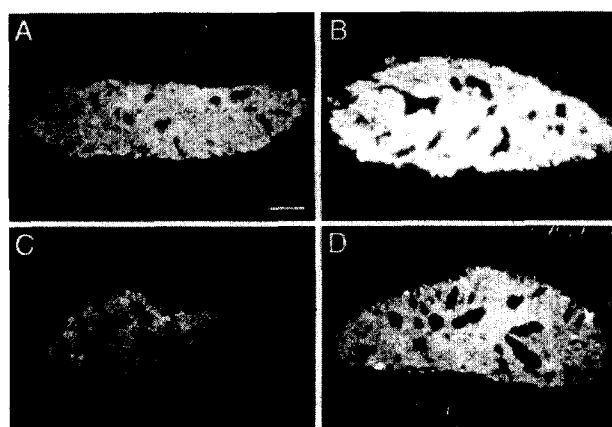


Fig. 2. The effects of reserpine on mRNA encoding TH in intact and splanchnicotomized adrenal medulla. After in situ hybridization using an  $^{35}\text{S}$ -labeled probe for rat TH mRNA, digital photomicrographs were prepared as described in the legend to Fig. 1. Micrographs representing each experimental condition are shown: (A) Intact-vehicle (1 ml/kg 20% ascorbic acid, s.c., 24 h prior to perfusion); (B) intact-reserpine (10 mg/kg s.c., 24 h prior to perfusion); (C) splanchnicotomy-vehicle; (D) splanchnicotomy-reserpine. Scale bar = 100  $\mu\text{m}$ .

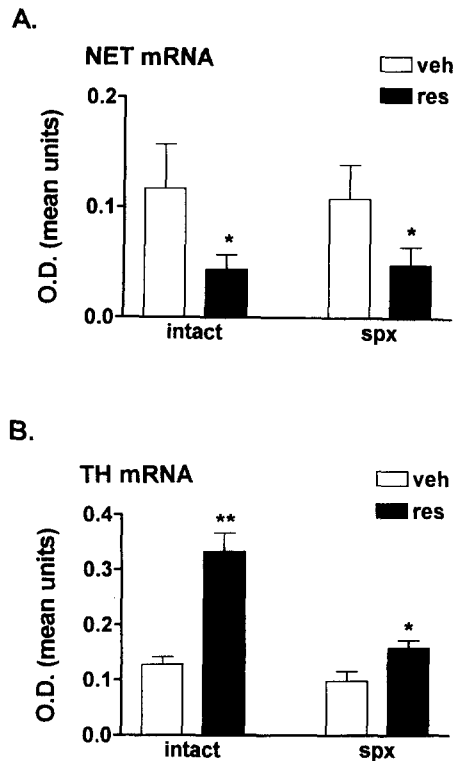


Fig. 3. Quantitative in situ hybridization analysis of mRNA levels in intact and splanchnicotomized (spx) adrenal medulla. After in situ hybridization using  $^{35}\text{S}$ -labeled probes for rat NET mRNA (A) or rat TH mRNA (B), optical densities (OD) were determined from autoradiograms of adrenal glands. OD values for adrenal medullas were calculated by subtracting background OD from adjacent adrenal cortices. (A) The effects of reserpine on mRNA encoding the NET. Mean OD values were calculated from averaged replicate OD determinations from 4–14 sections per adrenal gland, from 5 rats perfused 24 h after 10 mg/kg reserpine (res) or 5 rats perfused 24 h after 1 ml/kg of 20% ascorbic acid (veh). (B) The effects of reserpine on mRNA encoding TH. Mean OD values were calculated from averaged replicate determinations from 4–14 sections per adrenal gland, from 6 rats perfused 24 h after 10 mg/kg reserpine (res) or 6 rats perfused 24 h after 1 ml/kg of 20% ascorbic acid (veh). \* $P < 0.05$ , compared to the corresponding vehicle-treated group. \*\* $P < 0.001$ , compared either to the vehicle-intact group, or the reserpine-splanchnicotomy group.

serpine-treated intact adrenal medullas, the mean optical density (OD) from NET in situ hybridization was 63% lower than the mean OD from vehicle-treated intact adrenal medullas (2-way ANOVA, main effect of drug:  $F_{1,15} = 6.0$ ;  $P < 0.05$ ; Figs. 1A,B and 3A). Contrary to our expectation, splanchnicotomy did not alter the effect of reserpine on levels of NET mRNA in the adrenal medulla (Figs. 1B,D and 3A).

Consistent with results from many previous laboratories [2,4,10,19] reserpine produced a large (approximately 2.6-fold) increase in the mean OD of intact adrenal medullas processed for TH in situ hybridization, compared to intact adrenal medullas from rats given vehicle (2-way ANOVA, main effect of drug,  $F_{1,20} = 36.4$ ;  $P < 0.0001$ ; Figs. 2A,B and 3B). Splanchnicotomy substantially diminished the effect of reserpine on TH mRNA in the ad-

renal medulla (Figs. 2B,D and 3B), as revealed by significant main effects of surgery ( $F_{1,20} = 21.7$ ;  $P < 0.001$ ) and a significant drug-surgery interaction ( $F_{1,20} = 10.8$ ;  $P < 0.005$ ). Inspection of the quantitative data (Fig. 3B) shows the effect of reserpine on mRNA encoding TH is larger in the intact adrenal medullas than in the denervated adrenal medullas. However, reserpine also significantly increased levels of TH mRNA in the denervated adrenal medulla compared to the vehicle-treated denervated adrenal medulla ( $P < 0.05$ , post-hoc two-tailed  $t$ -test). In vehicle-treated adrenal medullas, splanchnicotomy by itself did not significantly alter TH mRNA levels ( $P > 0.1$ ).

The results reported here rule out the hypothesis that a trans-synaptic mechanism mediates the effect of reserpine on levels of mRNA encoding the NET. The presynaptic fibers innervating the adrenal medulla therefore do not carry the signal responsible for triggering the decrease in NET mRNA after administration of reserpine. Whether enhanced release of corticosteroids from the adrenal cortex triggers the reserpine-induced decrease in NET mRNA expression, as appears to be the case for stress-induced increases in adrenomedullary mRNA encoding phenylethanolamine *N*-methyl transferase [18], or some other signal is responsible, awaits further investigation.

The effect of reserpine on NET mRNA in the adrenal medulla was not altered by splanchnicotomy. In contrast, splanchnicotomy markedly diminished the effect of reserpine on adrenomedullary TH mRNA. A trans-synaptic mechanism therefore does not serve as a common signal coordinating the response of adrenomedullary TH and NET gene expression. In light of our observation that reserpine administration produces a significant increase in levels of adrenomedullary TH mRNA in denervated adrenal glands, we cannot exclude the possibility that one signal elicits the decrease in adrenomedullary NET mRNA and the innervation-independent component of the increase in TH mRNA. One strategy for identifying mechanisms capable of regulating both the NET and TH genes will be to clone and characterize the promoter of the NET gene, and compare it to that of the TH gene, about which much is already known [e.g. 3,6,7,11,20].

To our knowledge, the present experiments provide the first evidence that an innervation-independent mechanism mediates part of the induction of TH mRNA by reserpine in the adrenal medulla, in vivo. This finding is consistent with the observation that addition of reserpine to the medium increases levels of TH mRNA in cultured bovine chromaffin cells [16]. However, in hamsters, even high doses of reserpine do not increase adrenomedullary TH mRNA in denervated adrenals [5]. This discrepancy with our results could arise from interspecies differences in regulation of adrenomedullary gene expression, as has been demonstrated for mRNA encoding proenkephalin [5]. A previous investigation of the induction of TH

mRNA in the rat superior cervical ganglion did not detect an effect of reserpine on levels of TH mRNA in denervated ganglia [1]. It is possible that the different drug regimen employed in the previous study (5 mg/kg reserpine daily for 2 days, followed by sacrifice on the 3rd day), compared to the single 10 mg/kg dose employed in the present study, accounts for the different findings. Alternatively, small changes in TH mRNA may have been more difficult to resolve in that study because it analyzed pooled ganglia, whereas the present study analyzed individual adrenal glands from paired rats. Finally, the mechanisms underlying induction of TH in the chromaffin cells of the adrenal medulla and the neurons of the superior cervical ganglion may not be identical.

Immobilization stress increases TH mRNA expression in the denervated adrenal medullas of hypophysectomized rats by 50–60% [13]. Thus, a portion of the post-immobilization increase in TH mRNA in the adrenal medulla depends neither on a trans-synaptic nor a corticosteroid-mediated signal. Further work will be necessary to determine whether reserpine induces the innervation-independent increase in adrenomedullary TH mRNA by enhancing the release of adrenal corticosteroids [9]; or as in the case of immobilization stress, by another (as yet unknown) mechanism.

Supported by a Reader's Digest Fellowship, Department of Psychiatry, Cornell University Medical College (J.F.C.), the Burke Institute for Medical Research (B.T.V.), and National Institutes of Health grants AG 09686 (H.B.), MH 00149 (G.P.S.), and MH 24285 (T.H.J.). The authors are grateful to Dr. Danielle Greenberg for help with the statistical analyses, and to Nan Min and Charles Carver for assistance in preparing the figures.

- [1] Black, I.B., Chikaraishi, D.M. and Lewis, E.J., Trans-synaptic increase in RNA coding for tyrosine hydroxylase in a rat sympathetic ganglion, *Brain Res.*, 339 (1985) 151–153.
- [2] Cubells, J.F., Kim, K.S., Baker, H., Volpe, B.T., Chung, Y., Houpt, T.A., Wessel, T.C. and Joh, T.H., Differential *in vivo* regulation of the mRNA encoding the norepinephrine transporter and tyrosine hydroxylase in rat adrenal medulla and locus ceruleus, *J. Neurochem.*, (1995) in press.
- [3] Dawson, S.J., Yoon, S.O., Chikaraishi, D.M., Lillycrop, K.A. and Latchman, D.S., The Oct-2 transcription factor represses tyrosine hydroxylase expression via a heptamer TAATGARAT-like motif in the gene promoter, *Nucleic Acids Res.*, 22 (1994) 1023–1028.
- [4] Faucon-Biguot, N., Buda, M., Lamouroux, A., Samolyk, D. and Mallet, J. Time course of the changes of TH mRNA in rat brain and adrenal medulla after a single injection of reserpine, *EMBO J.*, 5 (1986) 287–291.
- [5] Franklin, S.O., Zhu, Y.-S., Yoburn, B.C., and Inturrisi, C.E., Transsynaptic activity regulates proenkephalin and tyrosine hydroxylase gene expression and the response to reserpine in the hamster adrenal, *Mol. Pharmacol.*, 40 (1991) 515–522.
- [6] Kim, K.-S., Lee, M.K., Carroll, J. and Joh, T.H., Both the basal and inducible transcription of the tyrosine hydroxylase gene are dependent upon a cAMP response element, *J. Biol. Chem.*, 268 (1993) 15689–15695.
- [7] Kim, K.-S., Tinti, C., Song, B., Cubells, J.F., and Joh, T.H. Cyclic AMP-dependent protein kinase regulates basal and cyclic AMP-stimulated but not phorbol ester-stimulated transcription of the tyrosine hydroxylase gene, *J. Neurochem.*, 63 (1994) 834–842.
- [8] Lambert, R., *Surgery of the Digestive System in the Rat*, Charles C Thomas, Springfield, IL, 1966, pp. 473–476.
- [9] Lowy, M.T., Nash, F. and Meltzer, H.Y., Reserpine-induced DST nonsuppression in rats, *Biol. Psychiatr.*, 27 (1990) 546–548.
- [10] Mallet, J., Faucon Biguet, N., Buda, M., Lamouroux, A. and Samolyk, D., Detection and regulation of the tyrosine hydroxylase mRNA levels in rat adrenal medulla and brain tissues, *Cold Spring Harbor Symp. Quant. Biol.*, 48 (1983) 305–308.
- [11] Min, N., Joh, T.H., Kim, K.S., Peng, C. and Son, J.H., 5' upstream DNA sequence of the rat tyrosine hydroxylase gene directs high-level and tissue-specific expression to catecholaminergic neurons in the central nervous system of transgenic mice, *Mol. Brain Res.*, 27 (1994) 281–289.
- [12] Müller, R.A., Thoenen, H. and Axelrod, J., Increase in tyrosine hydroxylase activity after reserpine administration, *J. Pharmacol. Exp. Ther.*, 169 (1969) 74–79.
- [13] Nankova, B., Kvetnansky, R., McMahon, A., Viskupic, E., Hiremagalur, B., Frankle, G., Fukuhara, K., Kopin, I.J. and Sabban, E.L., Induction of tyrosine hydroxylase gene expression by a nonneural nonpituitary-mediated mechanism in immobilization stress, *Proc. Natl. Acad. Sci. USA*, 91 (1994) 5937–5941.
- [14] Perlman, R.L. and Role, L.R., The coordinate control of catecholamine secretion, synthesis and reuptake in chromaffin cells. In Ben-Jonathan, N., Bahr, J.M. and Weiner, R.I. (Eds.), *Catecholamines as Hormone Regulators*, Serono Symposia Publications, Vol. 18, Raven Press, New York, 1985, pp. 215–221.
- [15] Role, L.W. and Perlman, R.L., Catecholamine uptake into isolated adrenal chromaffin cells: inhibition of uptake by acetylcholine, *Neuroscience*, 10 (1983) 987–996.
- [16] Stachowiak, M.K., Hong, J.S. and Viveros, O.H., Coordinate and differential regulation of phenylethanolamine *N*-methyl transferase, tyrosine hydroxylase and proenkephalin mRNAs by neural and hormonal mechanisms in cultured bovine adrenal medullary cells, *Brain Res.*, 510 (1990) 277–288.
- [17] Thoenen, H., Müller, R.A. and Axelrod, J., Trans-synaptic induction of adrenal tyrosine hydroxylase, *J. Pharmacol. Exp. Ther.*, 169 (1969) 249–254.
- [18] Viskupic, E., Kvetnansky, R.K., Sabban, E.L., Fukuhara, K., Weise, V.K., Kopin, I.J. and Schwartz, J.P., Increase in rat adrenal phenylethanolamine *N*-methyltransferase mRNA level caused by immobilization stress depends on intact pituitary-adrenocortical axis, *J. Neurochem.*, 63 (1994) 808–814.
- [19] Wessel, T.C. and Joh, T.H., Parallel upregulation of catecholamine-synthesizing enzymes in rat brain and adrenal gland: effects of reserpine and correlation with immediate early gene expression, *Mol. Brain Res.*, 15 (1992) 349–360.
- [20] Yoon, S.O. and Chikaraishi, D.M., Tissue-specific transcription of the rat tyrosine hydroxylase gene requires synergy between an AP-1 motif and an overlapping E box-containing dyad, *Neuron*, 9 (1992) 55–67.