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MOLECULAR PHARMACOLOGY, 40:515–522

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Transsynaptic Activity Regulates Proenkephalin and Tyrosine Hydroxylase Gene Expression and the Response to Reserpine in the Hamster Adrenal

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Received April 26, 1991; Accepted June 28, 1991

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SUMMARY

Transsynaptic neurogenic activity and reserpine are two signals that cause the proenkephalin (Penk) gene to alter the levels of preproenkephalin (PPenk) mRNA and enkephalin-containing (EC) peptides. In the Syrian hamster adrenal, but not in rat adrenal, both of these signals appear to be positive activators of Penk gene expression. The separate and combined effects of reserpine and denervation on EC peptides and catecholamine systems were investigated in the adrenal of the hamster, a species with relatively high medullary PPenk mRNA and EC peptide levels. Unilateral adrenal denervation resulted in a rapid decrease in PPenk mRNA levels of 54% after 2 days, and by 11 days 90% of Penk mRNA had disappeared. After 4 days both EC peptide and PPenk mRNA levels fell in parallel, whereas total RNA and soluble protein levels were unchanged. Denervation had no effect on TH mRNA levels until 8 days after surgery, and after 11 days both TH mRNA and catecholamine levels had decreased by 35–45%. Reserpine produced a dose- and time-dependent depletion of EC peptides and catecholamines. One day after 5 mg/kg reserpine (given subcutaneously on each of 2 consecutive days), EC peptides were reduced by 80%, norepinephrine by 79%, and epinephrine by >95%. By 4 days after treatment, EC peptides and catecholamines slightly exceeded or had returned to control (concurrent vehicle treatment) values. PPenk mRNA levels, as measured by solution hybridization, were doubled (206 ± 17%, mean ± standard error) by day 4. Tyrosine hydroxylase (TH) mRNA levels were increased nearly 7-fold (686 ± 71%) 24 hr after the first reserpine dose and declined thereafter. Northern blot analysis demonstrated that reserpine did not alter the size of either PPenk or TH mRNAs. Size exclusion chromatography showed a small (20%) reserpine-induced increase in processing of high molecular weight Penk-like peptides. The effects of reserpine, which increases PPenk mRNA, EC peptides, and TH mRNA, were completely blocked by unilateral denervation, whereas the contralateral innervated gland showed the expected responses. The co-localized EC peptide and catecholamine systems, as reflected in their mRNAs, respond differently in both time sequence and magnitude to reserpine and to denervation. Our results support a critical role, *in vivo*, for transsynaptic mechanisms in the maintenance of the high levels of Penk gene expression in this species and for the positive activation (mediated by reflex neurogenic stimulation) of reserpine on Penk and TH gene expression.

EC peptides are found in the central nervous system and at several peripheral sites (1–3). The adrenal medulla is a major peripheral site for EC peptide biosynthesis, and adrenal EC peptides are co-localized in chromaffin cell granules and coreleased with adrenal catecholamines (4, 5). In addition, evidence of enkephalinergic nerve fibers has been found in the adrenal medulla of several species (6). Some indication of the physiological functions of the adrenal EC peptides has begun to emerge with reports of their possible role in some forms of stress-induced analgesia (7) and in the modulation of the effects of coreleased catecholamines (8, 9). Thus, the adrenal EC peptides may have hormonal as well as the neurotransmitter functions that they may subserve in the central nervous system. The potential usefulness of EC peptides in reducing pain, their putative roles in the stress response, and their proposed modulation of some catecholamine responses create a rationale for studies of those factors that regulate the levels of these peptides.

Of particular interest are signals, both physiological and pharmacological, that alter Penk gene expression and EC peptide biosynthesis. In the hamster, removal of transsynaptic activity and depolarizing influences by unilateral denervation of the adrenal results in a time-dependent ipsilateral decrease in EC peptide levels and a 50% reduction in PPenk mRNA levels by 8 days after surgery (10). In cultured bovine adrenal medullary cells, depolarizing stimuli increase PPenk mRNA

This work was supported in part by National Institute on Drug Abuse Grants DA-01457 and DA-04185 and National Institute on Drug Abuse Center Grant DA-05130.

ABBREVIATIONS: EC, enkephalin-containing; PPenk, preproenkephalin; Penk, proenkephalin; TH, tyrosine hydroxylase; SEC, size exclusion chromatography.

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and EC peptide levels (11, 12). Thus, it may be inferred that transsynaptic neurogenic activity is a positive activator of Penk gene expression in both of these species. In contrast, regulation of Penk gene expression in the rat adrenal is more complex, in that a loss of depolarizing stimuli by denervation or explanation to culture increases PPenk mRNA and EC peptides (13–18). Furthermore, the normal innervated hamster adrenal medulla contains levels of EC peptides and PPenk mRNA that are 400- and 90-fold greater, respectively, than those of the rat and in the range observed for nonrodent mammals (e.g., bovine adrenal) (10).

Reserpine, an antihypertensive drug, inhibits the uptake of catecholamines into adrenal chromaffin granules (19), and the resulting reserpine-induced depletion of co-localized catecholamines and EC peptides has been associated with the reflex stimulation of the splanchnic nerve and the subsequent induction of adrenal TH enzyme (tyrosine 3-monooxygenase, EC 1.14.16.2) and EC peptides (20–23). In bovine chromaffin cells, reserpine produces a dose-dependent increase in TH mRNA and PPenk mRNA (24) (also see Refs. 25 and 26). However, in rat adrenal, reserpine treatment results in an increase in TH mRNA but a decrease in PPenk mRNA (27–29). Thus, reserpine is a pharmacological signal that produces positive activation of TH and a positive or negative activation of PPenk gene expression, depending on the species. These observations suggest a major role for transsynaptic activity both in the maintenance of the normal steady state and in reserpine-induced alteration of Penk gene expression in the adrenal.

In this study, we utilized specific and sensitive solution hybridization techniques to examine the separate and combined effects of reserpine and denervation on PPenk and TH mRNA levels in the hamster adrenal. We also examined the effects of reserpine on adrenal EC peptide levels and processing and on adrenal catecholamine levels. Our results present a quantitative demonstration of the temporal characteristics of the *in vivo* decline in PPenk and TH mRNA levels as a consequence of adrenal denervation. We show, for the first time, that denervation blocks the *in vivo* reserpine-induced increase in adrenal PPenk and TH mRNA levels. These observations indicate that a primary site of action of reserpine is at the genomic level.

Materials and Methods

Male Syrian (Golden) hamsters (60–70 g) were purchased from either Charles River Inc. (Wilmington, MA) or SASCO Inc. (Omaha, NE). Sacrifice was by decapitation, at which time whole adrenals (or adrenal medullae when specified) were dissected and immediately homogenized in 1 ml of RNA extraction buffer (10, 15) or in 1 ml of EC peptide extraction medium (30, 10).

EC peptide extraction and determination. The extraction of EC peptides was performed as described previously (10, 15, 30), and the resulting acetic acid extract could be used for both EC peptides and catecholamines (10). EC peptide levels in these extracts were determined by radioimmunoassays (15, 30) for Met-enkephalin before and after proteolytic digestion with trypsin and carboxypeptidase B, which defined free and total EC peptides, respectively (10, 14). The proteolytic digestion procedure, which has been described (10, 15, 30), releases free Met-enkephalin from higher molecular weight precursors (31).

Total RNA extraction. Total RNA was isolated using a guanidine HCl-phenol extraction/ethanol precipitation procedure described previously (15). Tissues from 10 animals were pooled, extracted for RNA, and used for Northern blot analysis, whereas individual tissues from six animals were extracted and used for solution hybridization studies. When total RNA was extracted from individual adrenal glands, 50 μg of *Escherichia coli* tRNA carrier were added to the extraction buffer before homogenization and 20 μg of tRNA carrier were added to each subsequent ethanol precipitation step. The recovery of total RNA was 77.0 ± 7.2% (mean ± standard deviation) (15).

Northern blot. Northern blot analysis was performed, as previously described (15), on total RNA extracts that were denatured in 1 M glyoxal/50% (v/v) dimethyl sulfoxide for 60 min at 50°.

RNA probes. The ^{32}P-labeled riboprobes were transcribed from plasmids containing cDNAs for rat PPenk mRNA (15), human 18 S rRNA (15), or rat TH mRNA. The TH-containing plasmid (pGEM4) contains the 385-base-long sequence present in a *Eco*RI-*Kpn*I fragment from the TH cDNA of Grima *et al.* (32) (a gift from Drs. M. Evinger and T. Joh). The specific activities for both the PPenk and TH mRNA riboprobes[1] averaged 6.6×10^8 dpm/μg and that for the 18 S rRNA riboprobe was 1.0×10^7 dpm/μg.

Solution hybridization assay. This assay is based on the protection of ^{32}P-labeled riboprobes from ribonuclease digestion when they are hybridized to complementary RNAs (10). The protected riboprobe hybrids were precipitated with trichloroacetic acid and analyzed by scintillation counting. Quantitation of total RNA or mRNA was made by comparisons with standard calibration curves. For the quantitation of PPenk and TH mRNA levels, nonradiolabeled "sense" transcripts were synthesized *in vitro*, quantitated by UV spectra, and used as standards (15). Total rat liver RNA extracts were quantitated by UV spectra and used as standards for the 18 S rRNA determinations. A close agreement was found between the total cellular RNA values of samples from various tissues, including the adrenal from the hamster, when determined by both the solution hybridization assay and UV absorbance (correlation coefficient = 0.98). The riboprobe for rat PPenk mRNA used in this study contains sequences that are not complementary to hamster PPenk mRNA (10). Therefore, hamster PPenk mRNA values will be proportionally underestimated, and they are given as picogram equivalents. RNase protection assays demonstrate that RNA extracted from hamster adrenals protects a single TH mRNA fragment. The amount of the TH sense standards was multiplied by 5 to account for the difference in the length of the sense transcript and TH mRNA (approximately 423 bases and 1900 bases, respectively).

The calibration curves for the solution hybridization assays are linear as follows: from 2 to 250 pg for the PPenk mRNA assay, from 10 to 625 pg for the TH mRNA assay, and from 2.5 to 60 ng for the total RNA assay. The interassay coefficient of variation averaged 9.2% for 10 consecutive TH mRNA assays, and the interassay coefficient of variation averaged 5.1% for duplicate sample determinations. These assay characteristics are similar to those found for the assay of PPenk mRNA and total RNA (10).

Catecholamine determinations. Catecholamines are stable for at least 12 weeks at −20° in EC peptide extraction medium. These extracts were used to determine both norepinephrine and epinephrine levels, as previously described (10).

SEC. Adrenal medullary extracts were chromatographed on two 7.5-mm × 30-cm TSK high pressure liquid chromatography columns (TSK G2000 SW and G3000 SW) coupled in series (30). The column was eluted with 0.1% trifluoroacetic acid in 0.1 M NaCl, at a flow rate of 1 ml/min. Fractions of 1 ml were collected.

Reserpine treatment. Hamsters were injected subcutaneously once a day for 2 consecutive days with 1.25–10 mg/kg reserpine (Sigma, St. Louis, MO), using 0.31–2.5 mg/ml stock solutions of reserpine. This injectable form of reserpine was prepared in vehicle composed of 2.5 mg/ml anhydrous citric acid, 2% (v/v) benzyl alcohol, and 10% (v/v) Tween 80 (33).

Adrenal denervation. Hamsters were housed for 6 days before sectioning of the splanchnic nerve to the left adrenal gland, as previously described (10, 18). Care was taken to avoid surgically induced

[1] The terms "PPenk riboprobe," "TH riboprobe," and "18 S rRNA riboprobe" refer to ^{32}P-labeled RNA transcripts complementary to portions of PPenk mRNA, TH mRNA, and 18 S rRNA, respectively.

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damage to the adrenal vasculature. During dissection of the adrenal gland, gross examination did not indicate any signs of infarction or necrosis resulting from the denervation procedure. The innervated right gland from surgically treated hamsters and the adrenals from untreated hamsters were the controls. EC peptides and catecholamines in the individual glands were determined as described above. A separate set of glands were used to extract total cellular RNA, which was used for solution hybridization assays.

Statistics. One-way analysis of variance was used to determine significant treatment-induced changes. Subsequent treatment group comparisons were made using Duncan's multiple range test, with the level of significance at $p = 0.05$.

Results

Neurogenic regulation of adrenal EC peptide and catecholamine systems. Fig. 1 shows the time course of changes in PPenk mRNA, TH mRNA, and total cellular RNA after adrenal denervation, as measured by solution hybridization. For comparison, this figure also includes the time course of changes in EC peptide and epinephrine levels after denervation (data from Fig. 6 of Ref. 10). The results show a rapid and significant decline of 54% in PPenk mRNA by day 2 after adrenal denervation. This decline continued for at least 11 days after surgery, when PPenk mRNA levels had been reduced by approximately 90%. Concurrently, EC peptide levels fell more slowly, and they were not significantly reduced until 4 days after surgery. Thereafter, PPenk mRNA and EC peptide levels appeared to decline in parallel. TH mRNA levels remained unchanged in denervated adrenals at 4 days after surgery. These levels began to decline at 8 days, and they were significantly reduced by 45% at 11 days after surgery. The change in epinephrine levels was parallel to that for TH mRNA both in the time course and in the magnitude of the decline. Total RNA levels (Fig. 1) and total soluble protein levels (data not shown) were unchanged for at least 11 days after surgery.

Reserpine-induced depletion of adrenal EC peptides and catecholamines. Fig. 2 shows that reserpine produces a dose-dependent reduction in hamster adrenal EC peptides and catecholamines. Normal hamsters were given two subcutaneous injections of reserpine or vehicle, 24 hr apart. Twenty-four

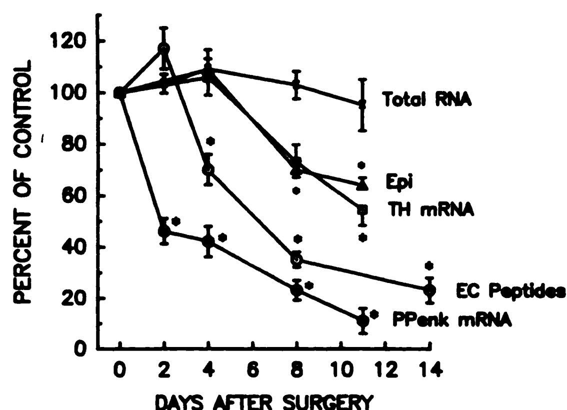

Fig. 1. Effect of denervation on mean ± standard error (N = 6) epinephrine, TH mRNA, EC peptide, and PPenk mRNA levels in hamster adrenal. Day 0 values are the values for untreated hamsters, and the values after surgery are the percentage obtained by dividing the values for denervated adrenals by the corresponding values for innervated adrenals. *, Significantly different for innervated adrenal. *Epi*, epinephrine; *Norepi*, norepinephrine; *Total RNA*, total cellular RNA. The epinephrine and EC peptide data are taken from Fig. 6 of Ref. 10.

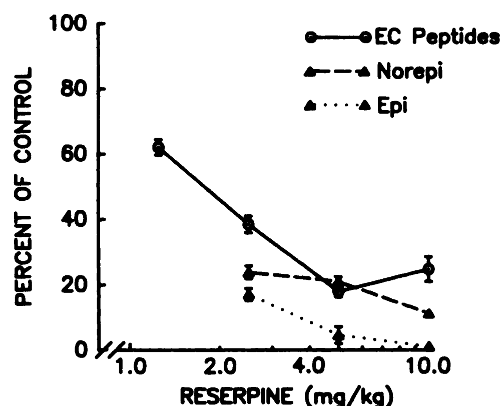

Fig. 2. Dose-response relationship for the reserpine-induced decrease in hamster adrenal medullary EC peptides and catecholamines. The reserpine treatment schedule was once a day for 2 consecutive days, and the subcutaneous dose is shown on the *abscissa*. The response was measured 24 hr after the second dose. The data are the mean percentage effect ± standard error (N = 6), obtained by dividing values for reserpine-treated animals by the corresponding values for vehicle-treated animals. Abbreviations are as in Fig. 1.

hours after the second injection, these hamsters were sacrificed and total EC peptide and catecholamine levels in adrenal extracts were determined. With the highest reserpine doses (5 or 10 mg/kg), adrenal EC peptide levels were reduced approximately 80%, to about 30 pmol of Met-enkephalin equivalents/gland. Catecholamine levels were also significantly reduced, with norepinephrine levels that were 21% of vehicle and epinephrine levels that were <5% of vehicle. With this dosing schedule, the ED_{50} for the reduction of EC peptides was equal to 1.89 mg/kg (the 95% confidence interval was 1.58–2.24 mg/kg and the slope was 3.5). Because the highest dose tested (10 mg/kg) did not produce additional depletion in EC peptide levels, we chose two consecutive daily doses of 5 mg/kg, as previously used in rats (21), for further studies in hamsters.

Northern blot analysis of total RNA from the adrenals of reserpine-treated hamsters. Northern blot analysis (Fig. 3) was performed on total RNA extracts of adrenals from vehicle-treated hamsters and from hamsters treated with two doses of 2.5, 5, or 10 mg/kg reserpine, as described above. Animals were sacrificed at the time of peak EC peptide depletion, i.e., 24 hr after the second injection (see Fig. 2 and below). Separate filters were hybridized with riboprobes for PPenk mRNA (Fig. 3A) or TH mRNA (Fig. 3B). Although some small changes in the level of PPenk mRNA were evident using autoradiographic analysis, we did not attempt to quantitate these changes here; rather, we used the Northern blot analysis to demonstrate that no alteration occurred in the size of the PPenk mRNA (approximately 1500 bases; see Ref. 10) as a result of reserpine treatment.

Fig. 3B shows a very large increase in the abundance of TH mRNA in adrenal extracts from reserpine-treated (2 × 5 mg/kg) hamsters, compared with vehicle-treated animals. Examination of the Northern blot indicates that reserpine had no effect on the size of hamster adrenal TH mRNA (approximately 1900 bases). Furthermore, the size estimates for PPenk mRNA and TH mRNA obtained by Northern blot analysis of hamster adrenal RNA extracts are virtually identical to those reported for the rat (10, 28).

Time-course of reserpine-induced effects. Fig. 4 shows the time-course of reserpine-induced changes in TH and PPenk

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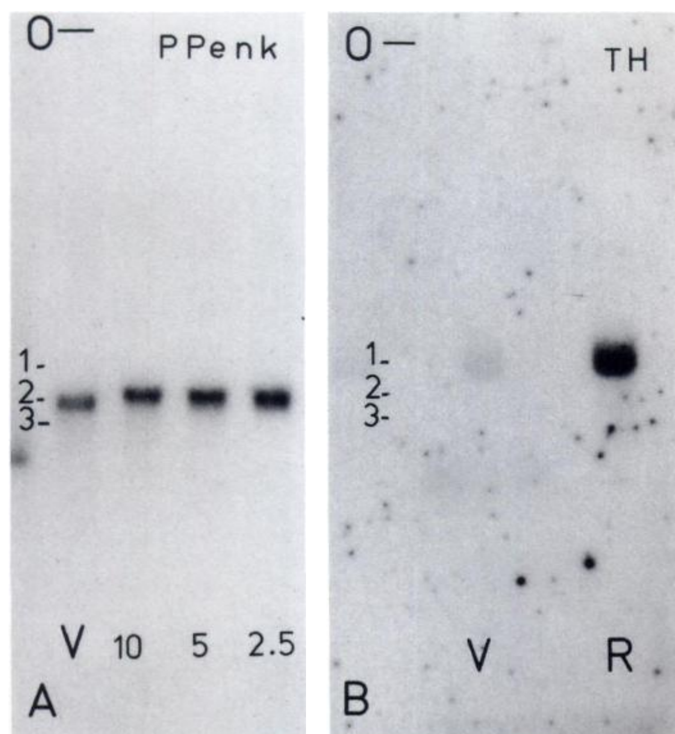

Fig. 3. Northern blot analysis of PPenk (A) and TH (B) mRNA in total RNA extracts of adrenal tissues from hamsters 24 hr after treatment. RNA aliquots were 7 μg in A and 2 μg in B. *0*, origin of electrophoresis; *positions 1*, *2*, and *3*, migration of markers with sizes of 1904, 1584, and 1330 bases, respectively. *V*, vehicle treatment; *R*, reserpine treatment. In A, reserpine treatment was once a day for 2 consecutive days and the subcutaneous doses were 10, 5, or 2.5 mg/kg. In B, the reserpine treatment schedule was as in A, with a subcutaneous dose of 5 mg/kg.

mRNA levels in the hamster adrenal, using quantitative solution hybridization methods (Fig. 4A). Fig. 4B presents the change in hamster adrenal EC peptide and catecholamine levels after reserpine treatment (2 × 5 mg/kg, subcutaneously). Hamsters were sacrificed on day 0 (24 hr after the first injection, just before the second injection) and on days 1, 4, and 12 after the second injection. From days 0 to 4 there was a steady increase in PPenk mRNA levels, until they peaked at 2-fold over control on day 4. PPenk mRNA levels remained slightly but significantly elevated (1.4-fold) at 12 days after treatment. The time course for the change in PPenk mRNA was very different from that observed with TH mRNA. TH mRNA levels increased to nearly 7-fold over control 24 hr after the first injection (day 0) and progressively declined to levels equal to control by day 12.

The maximum depletion of EC peptide and catecholamine levels was observed 1 day after the second reserpine dose, and the magnitude of these decreases was similar to that seen earlier (see Fig. 2). By 4 days after reserpine treatment EC peptide levels had increased above control levels, and this small but significant increase, of approximately 25%, was sustained at least 12 days after treatment. Norepinephrine levels returned to control levels by 4 days and remained at this level at 12 days after treatment. Epinephrine levels appeared to lag in their recovery phase, but they returned to vehicle control levels by 12 days after treatment.

Fig. 5 shows the results of SEC under conditions that provide for the rapid separation of high (tubes 17–21), intermediate (tubes 22–26), and low (tubes 27–35) molecular weight EC peptides (10, 14, 30). Penk found in cultured rat medullary explants (30) and the Penk-like peptides found in extracts from hamster adrenal medullae (10) are eluted predominantly in the high molecular weight tubes. The SEC profiles are derived from aliquots from pooled adrenal medullary extracts from hamsters 4 days after vehicle (Fig. 5A) or reserpine treatment (Fig. 5B). The fraction of high molecular weight EC peptides (tubes 17–21) was reduced from 98% to 78% by reserpine treatment, whereas the fraction of intermediate molecular weight EC peptides (tubes 22–26) increased from 1 to 19% and the low molecular weight fraction (tubes 27–35) increased from 1 to 3%. The contents of these latter fractions represent approximate estimates, because of the low amounts of EC peptides present. These profiles indicated that, after recovery from reserpine-induced depletion, there are apparent increases in the prevalence of intermediate molecular weight EC peptides. Nevertheless, the major EC peptides in the adrenals from both reserpine- and vehicle-treated hamsters are of high molecular weight.

Using Western blot analysis, we reported (10) the presence in hamster adrenal medulla of two Penk-like peptide bands, one with a molecular weight of 34,000 (major band) and the other at 37,500. Both of these bands (and no other bands) are found in adrenal extracts from reserpine- and vehicle-treated hamsters (data not shown).

Effects of reserpine and the role of innervation in the adrenal. Figs. 6, 7, and 8 show the effects of reserpine on PPenk mRNA, EC peptides, and TH mRNA in the denervated and innervated adrenals from individual hamsters. The inclusion of an assay for ribosomal RNA serves to provide an internal standard for the RNA extraction and recovery steps and allows normalization of PPenk and TH mRNA values from different samples. Reserpine treatment was initiated 6 days after surgery, and observations were made 8 and 11 days after surgery. This period was selected because the effects of surgery on both EC peptides and catecholamines appear to be near maximum at this time (see Fig. 1). In denervated adrenals, reserpine failed to alter PPenk mRNA levels, compared with vehicle, at either 1 or 4 days after treatment. However, reserpine produced significant increases in PPenk mRNA levels in the innervated glands from these same animals on day 4 after treatment (see also Fig. 4A). Total RNA levels were not altered in denervated or innervated adrenals by reserpine or vehicle treatment (data not shown).

Fig. 7 compares the EC peptide levels in the innervated and denervated adrenals from reserpine-treated hamsters (4 days after treatment) with those in untreated controls. Reserpine treatment significantly increased EC peptide levels in the innervated adrenals (see also Fig. 4B). However, EC peptide levels in the denervated adrenals were significantly reduced from those found in the innervated glands from reserpine-treated hamsters and in glands from untreated hamsters.

To provide for a direct comparison between PPenk and TH mRNA levels, the samples utilized in Fig. 6 were also analyzed for TH mRNA, and the results are presented in Fig. 8. TH mRNA levels in the denervated adrenals from reserpine-treated hamsters were not altered at 1 and 4 days after treatment, compared with glands from vehicle-treated hamsters. However, as shown in Fig. 4A, reserpine treatment increased TH mRNA levels in the innervated contralateral adrenals on both day 1 and 4 after treatment.

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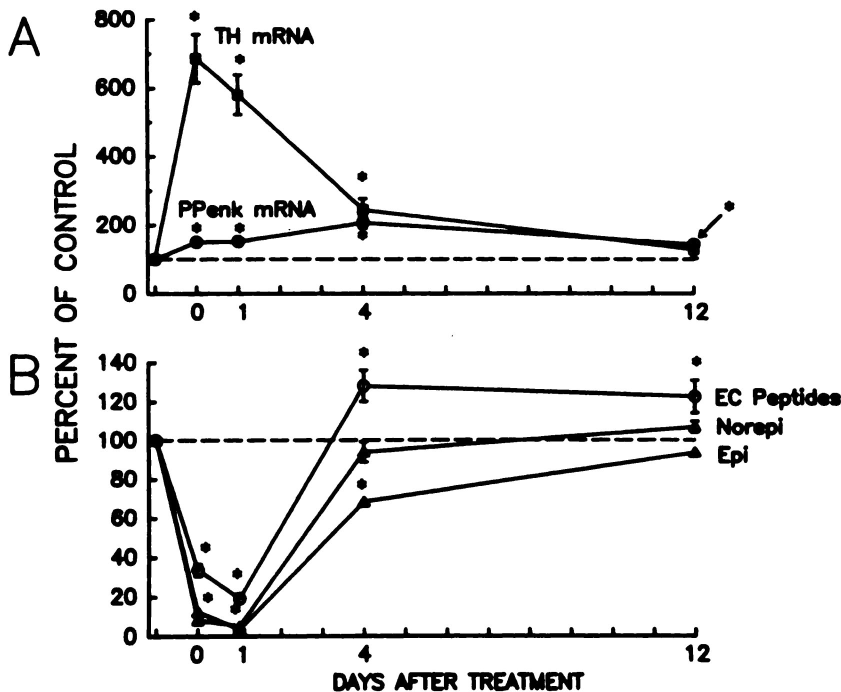

Fig. 4. Time course of reserpine-induced changes in PPenk mRNA and TH mRNA (A) and EC peptides, norepinephrine, and epinephrine levels (B). The reserpine treatment schedule was once a day for 2 consecutive days, with a subcutaneous dose of 5 mg/kg. Day 0 refers to 24 hr after the first dose, and days 1 to 12 refer to time after the second dose of reserpine. The data are the mean ± standard error (N = 8) obtained by dividing values for reserpine-treated animals by the corresponding values for vehicle-treated animals. *, Significantly different from vehicle. Abbreviations are as in Fig. 1.

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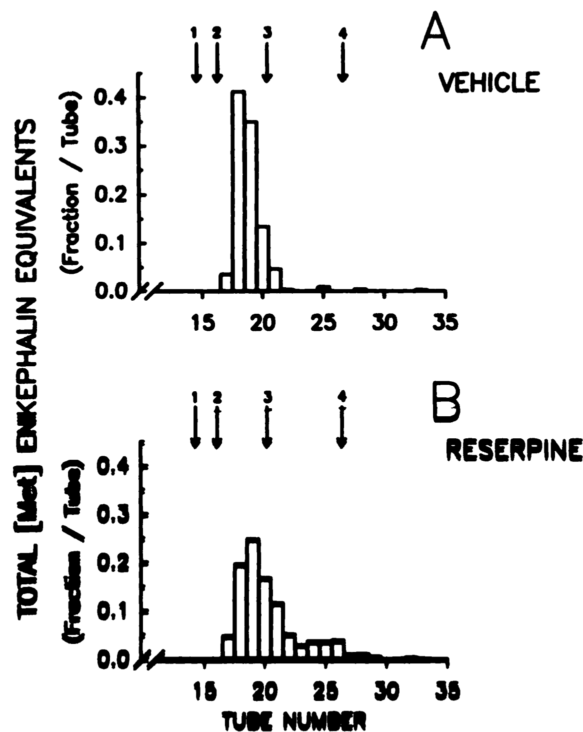

Fig. 5. SEC of adrenal medullary extracts from hamster. Reserpine and vehicle treatment were as in the legend to Fig. 4. Eighteen adrenal medullae were collected 4 days after the reserpine treatment was completed. The 1-ml fractions were assayed for total Met-enkephalin and the results are presented as the fraction of Met-enkephalin equivalents recovered in each tube. *1–4*, markers for retention characteristics of the column, representing albumin, carbonic anhydrase, cytochrome *c*, and phenylalanine, respectively.

Discussion

In this report, we extend our previous findings that demonstrate that transsynaptic activity is necessary for the maintenance of relatively high steady state levels of EC peptides in the hamster adrenal. This was done by describing the effects of unilateral denervation on selected Penk and TH phenotypic characters. The rapid and sustained decrease in PPenk mRNA that precedes the fall in EC peptides (Fig. 1) strongly supports the conclusion that transsynaptic activity and depolarizing influences are a positive regulator of Penk gene expression. In the adrenal, transsynaptic activity results in the activation of cholinergic receptors (34, 35). In the bovine chromaffin cell, nicotinic receptor activation initiates the coupled sequence of stimulus-secretion-synthesis of EC peptides and catecholamines (26). These processes involve calcium ion and cAMP, and both of these signal transduction mechanisms may be involved in the activation of PPenk and TH gene expression (26). Our *in vivo* experiments were not designed to determine whether the predominant effect of an intact nerve is on the transcription of the Penk gene or on the stabilization of mRNAs. If the denervation-induced decline in PPenk mRNA is due to the absence of Penk gene transcription, then a calculation of PPenk mRNA disappearance kinetics can be attempted. A semilogarithmic plot of the data in Fig. 1 reveals a monoexpotential decline in PPenk mRNA, with a $t_{1/2}$ of 3.1 days (correlation coefficient = 0.97). However, without time points earlier than 2 days, the contribution of a rapid initial phase to the disappearance kinetics of PPenk mRNA cannot be assessed. A denervation-induced change in EC peptides at 2 days after surgery is obscured by a surgically induced decrease in both denervated and innervated adrenals (see Ref. 10). After 2 days, the decline in EC peptides clearly parallels the decline in PPenk mRNA (Fig. 1). This indicates a tight sequential coupling between changes in PPenk mRNA and EC peptides, which is consistent with transcriptional control and/or mRNA stabilization of hamster adrenal EC peptides. The total to free ratio of EC peptides in denervated hamster adrenals is unchanged (data not shown), suggesting that denervation does not alter processing and that the predominant EC peptide in denervated adrenal remains Penk-like (10).

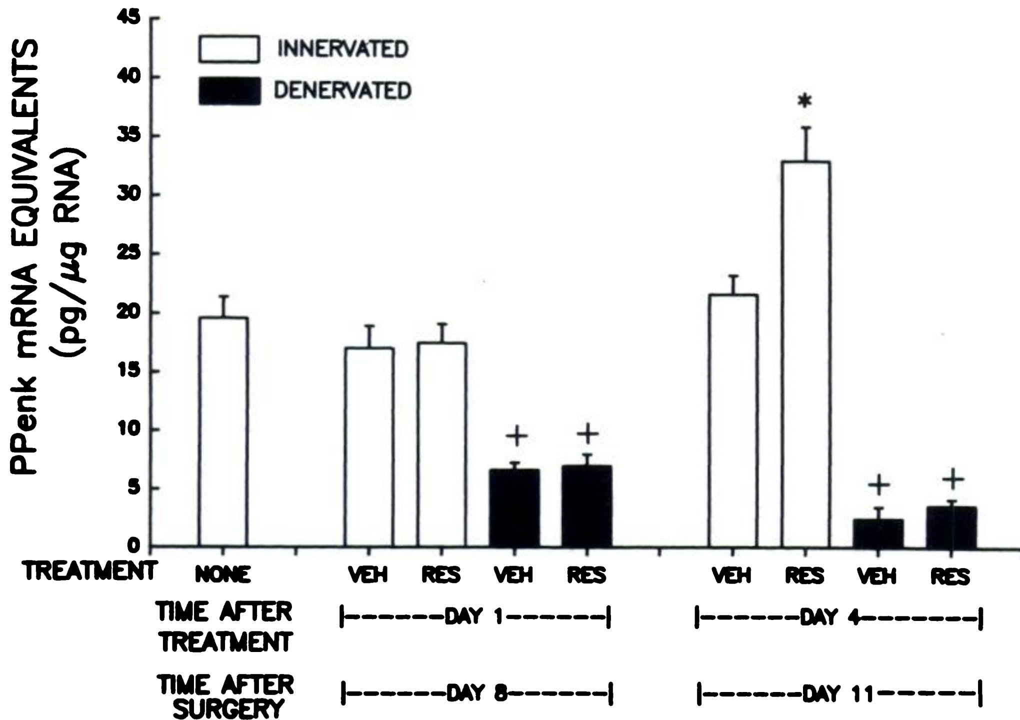

Fig. 6. Effect of reserpine treatment on PPenk mRNA levels in innervated and denervated hamster adrenals. The data are the mean ± standard error (N = 6). Unilateral adrenal denervation was performed 6 days before reserpine treatment, which was as described in the legend to Fig. 4. Measurements were made at 8 and 11 days after surgery, which correspond to 1 and 4 days after reserpine treatment was completed. *NONE*, untreated; *VEH*, vehicle; *RES*, reserpine; +, significantly different from innervated; *, significantly different from vehicle.

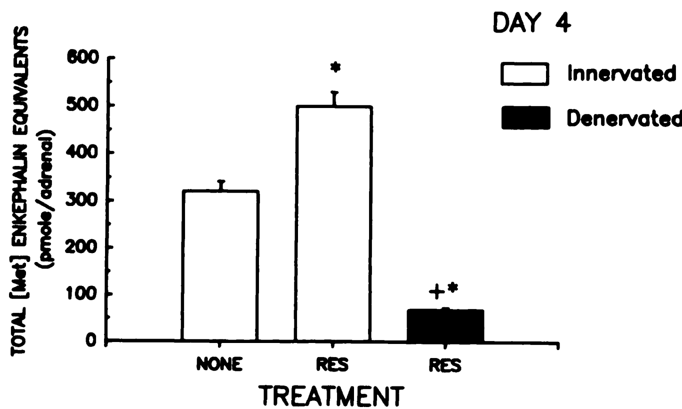

Fig. 7. Effect of reserpine treatment on EC peptide (total Met-enkephalin equivalents) levels in innervated and denervated hamster adrenals. The data are the mean ± standard error (N = 6). Surgery and reserpine treatment were as described in the legend to Fig. 6. Day 4 corresponds to 11 days after surgery and 4 days after reserpine treatment was completed. *NONE*, untreated; *RES*, reserpine treatment; *, significantly different from none; +, significantly different from innervated.

The other measured effects of denervation are much less dramatic and delayed, compared with effects on the Penk system. During the 11 days after denervation, total RNA levels (Fig. 1) and total soluble protein levels[2] are unchanged. Because the adrenal medulla comprises <10% of the weight, protein, and RNA content of the whole gland, the failure to detect a change in these values does not exclude alterations localized to the adrenal medulla. Catecholamine and TH mRNA do not decline until 8–11 days after surgery and then by only 35–45% of control. These results suggest that the catecholamine biosynthetic system is much less dependent on an intact nerve and transsynaptic influences. This has also been shown for the rat adrenal (21, 35). In rat preganglionic sympathetic neurons, Zigmond and Bowers (36) directly compared the relationship between nerve activity and TH enzyme activity, using electrical stimulation. They concluded that the very low intensity of spontaneous nerve activity is insufficient to influence TH activity. If this relationship exists in the adrenals, it could explain why denervation produces little if any change in TH activity (36) and only a moderate reduction in catecholamines (Fig. 1). These observations are also consistent with the concept that the adrenal Penk and catecholamine systems are regulated differently by transsynaptic influences (17, 23), even though EC peptides and catecholamines are co-localized in chromaffin granules (4, 5). In this regard, it is of interest to note that Viveros *et al.* (37) have suggested that the increase in EC peptides that occurs in rat adrenal medulla after denervation or explantation is secondary to the observed decrease in adrenal catecholamines. Our results in the hamster do not support this model, because at 8–11 days after surgery we see a decrease in both catecholamines and EC peptides (Fig. 1).

From studies in a number of species, reserpine has been shown to produce a dose dependent depletion of catecholamines (38). A dual mechanism of action is postulated, whereby at lower doses the reserpine-induced depletion of catecholamines is due mainly to inhibition of the uptake mechanism into chromaffin granules (38). This depletion does not require an intact nerve (39). At higher doses, the effects of reserpine result from a combination of uptake inhibition and neurogenic stimulation, which results in exocytosis of the entire soluble content of the storage vesicle and a more rapid depletion of catecholamines (38). The latter mechanism requires an intact nerve supply (21, 38). It has been hypothesized that reserpine produces a reflex increase in preganglionic activity of the sympathoadrenal system (21). Although the mechanism of this reflex activity is not well understood, it is clear that direct stimulation of preganglionic sympathetic nerves (e.g., to the rat superior cervical ganglion) results in increases in TH activity similar to that seen after reserpine (36). Because we were interested in the role of transsynaptic activity on Penk gene expression, we

[2] S. O. Franklin, unpublished observations.

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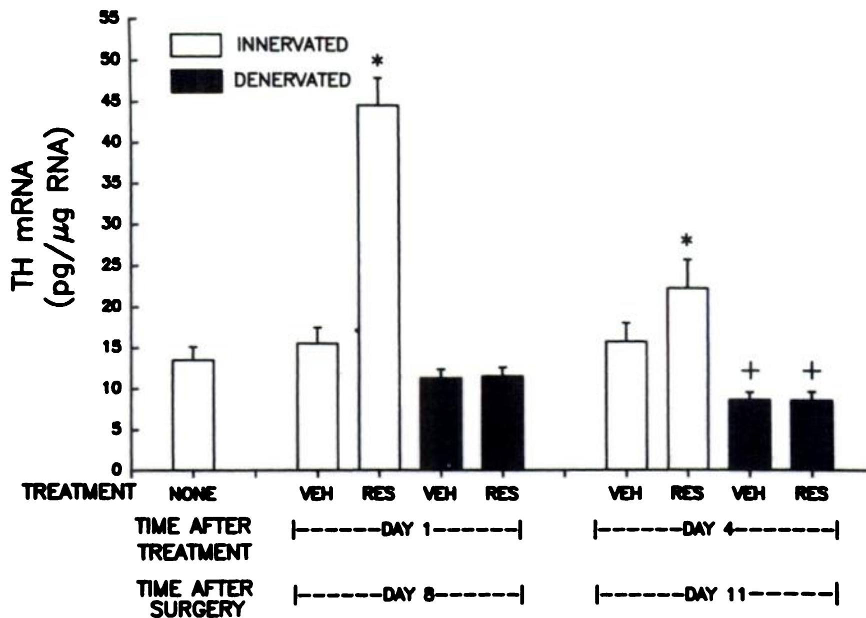

Fig. 8. Effect of reserpine treatment on TH mRNA levels in innervated and denervated hamster adrenals. The data are the mean ± standard error (N = 6). These are the same samples used for the measurement of PPenk mRNA shown in Fig. 6. +, Significantly different from innervated; *, significantly different from corresponding vehicle controls. Abbreviations are as in the legend to Fig. 6.

selected a dosing schedule for reserpine treatment that produced a near-maximal depletion of both EC peptides and catecholamines (Fig. 4). The time-course of reserpine-induced depletion and the return to pretreatment levels for EC peptides and catecholamines in hamster adrenal (Fig. 4) is similar to that observed in other species (23, 38).

In the rat adrenal, the reserpine-induced depletion of the constituents of chromaffin granules induces the synthesis of EC peptides, enzymes, and chromogranins (23) at independent rates, so that chromaffin granules are produced with significantly altered compositions, compared with the untreated state (23). In addition to its effects on mRNA abundance, reserpine increases EC peptide-processing enzymes (40, 41) and the posttranslational processing of the Penk precursors in rat adrenal and bovine chromaffin cells (25, 29, 42). We have observed both an apparent reserpine-induced increase in EC peptide processing (Fig. 5) and an increase in the levels of Penk precursor peptide (Fig. 7). We also noted a small but significant increase in PPenk mRNA, which reached 2-fold by 4 days after reserpine treatment. This increase in PPenk mRNA follows the reserpine-induced depletion of EC peptides.

The effects of reserpine *in vivo* on PPenk mRNA are undoubtedly dose dependent, because Mocchetti *et al.* (29) administered a dose of reserpine to rats that did not decrease EC peptides and, therefore, they observed only the presumed consequences of increased Penk processing and a feedback reduction of PPenk mRNA. Although our data are limited, it would appear that PPenk mRNA levels are decreased 12 days after reserpine treatment, compared with the 4-day values, whereas EC peptide levels remain elevated (Fig. 4). Thus, our data are consistent with a modest reserpine-induced increase in Penk gene expression in the hamster. The time-course of reserpine effects on TH mRNA differs from that of PPenk mRNA in several respects. The TH mRNA rise was earlier, much higher, and of much shorter duration than the concurrent profile for PPenk mRNA. A similar time-course for TH mRNA is seen in the rat adrenal after reserpine (28), and a lag in the induction of PPenk mRNA, compared with TH mRNA, is observed in chromaffin cells exposed to reserpine (24).

Unilateral denervation provides the opportunity to investigate the roles of transsynaptic mediation in PPenk and TH gene expression. In the rat, denervation did not change catecholamines (21) or they were reduced (14), whereas TH activity (21) and neuropeptide Y mRNA abundance (43) were unchanged. However, denervation prevented the reserpine-induced increases in both TH activity (21) and neuropeptide Y mRNA abundance (43). Consistent with these observations, we found that after adrenal denervation reserpine was unable to increase the levels of PPenk mRNA (Fig. 6), EC peptides (Fig. 7), or TH mRNA (Fig. 8) in hamster adrenal, although reserpine-induced increases were observed in the contralateral intact gland (Figs. 6–8). Reserpine produces temporal and quantitative differences in the induction of PPenk mRNA and TH mRNA (Fig. 4), and denervation prevented each of these effects of reserpine. Furthermore, denervation blocked the effects of reserpine regardless of whether denervation produced a reduction (PPenk mRNA) or no change (TH mRNA) in steady state levels (Fig. 6 and 8). These results indicate that, in the hamster adrenal, reserpine at the dosage used in this study alters Penk and TH gene expression by a transsynaptically mediated mechanism.

Depolarizing conditions increase bovine chromaffin EC peptides, suggesting that, like the hamster gene, the bovine Penk gene *in vivo* is under positive regulation by transsynaptic activity (26). Regulation of the rat adrenal Penk gene appears to be more complex, with tonic inhibition being the primary consequence of transsynaptic activity (13–18). These species-dependent differences in adrenal Penk gene regulation result in a much greater abundance of PPenk mRNA and higher levels of EC peptides in both hamster and bovine adrenal compared with rat (10). Yet much needs to be learned as to the species-and/or tissue-specific factors responsible for the high steady state levels of hamster adrenal EC peptides and PPenk mRNA. To provide some comparative information on genomic regulatory sites, we have isolated genomic Penk clones from the hamster and are sequencing the 5′ upstream portion of the gene.[3] Determination of the physiological consequences of these

[3]Y.-S. Zhu *et al.*, unpublished observations.

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differences in Penk gene expression also awaits a more complete definition of the role of adrenal EC peptides in stress and pain modulation. It would, however, appear that comparisons linking biochemical and behavioral relationships could be facilitated by comparing rat and hamster adrenal EC peptide systems. A combination of *in vivo* pharmacological studies and molecular structural experiments should provide important new insights into Penk gene regulation and the physiological and behavioral consequences.

Acknowledgments

We thank Daniel Cerbone and Theresa Huang for their skillful technical assistance.

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