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Time course of the changes of TH mRNA in rat brain and adrenal medulla after a single injection of reserpine

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A single injection of reserpine causes a long lasting enhancement of the activity of tyrosine hydroxylase (TH), the enzyme catalyzing the rate-limiting step in the biosynthesis of catecholamines. A sensitive method has been developed to assay both TH mRNA level and enzyme activity in tissue from a single rat. The time course of the induction was analysed in adrenals, locus coeruleus and substantia nigra. In both locus coeruleus and adrenals reserpine caused respectively 4.2- and 4.5-fold increase of TH mRNA which was maximal 2 days after drug injection. This increase is about twice that of the enzyme activity. No change was observed in substantia nigra. The effect lasted longer in locus coeruleus than in adrenal. In the latter, TH mRNA had almost returned to initial values at day 4 whereas at this time it is 3-fold higher in locus coeruleus and still significant at day 18. This result suggests that induction of TH results from an enhanced transcription of the TH gene. The time course difference between locus coeruleus and adrenals is most likely to result from a difference in the stability of TH mRNA in the two structures.

Key words: tyrosine hydroxylase induction/mRNA accumulation/tyrosine hydroxylase

Introduction

In addition to generating changes in electrophysiological properties of neuronal cells, synaptic stimulation fosters a variety of biochemical modifications that may well form the molecular basis of some aspects of the plasticity that characterizes brain structure and function. One of the best documented examples concerns the enzyme tyrosine hydroxylase (TH) which is the rate-limiting enzyme in the synthesis of catecholamines and plays a key role in the physiology of adrenergic neurons. Its expression is modulated in the developing and adult organism, both by short-term and long-term regulation (Zigmond, 1985).

Stimulations such as cold stress, or the administration of reserpine cause a 2- to 4-fold increase of TH activity (Thoenen *et al.*, 1969; Theonen, 1970; Joh *et al.*, 1973; Reis *et al.*, 1974, Zigmond, 1979). The rise in enzyme activity reaches a maximum level 48–72 h after stimulation and lasts as long as 3 weeks. Interruption of the afferent nerves in adrenals and superior cervical ganglia abolished the effect which has, therefore, been referred to as trans-synaptic induction. The increase in TH activity that occurs in adrenal medulla and locus coeruleus following administration of reserpine has been shown by immunoprecipitation studies to result from an increase in the amount of TH and not from an activation of pre-existing enzyme molecules (Joh *et al.*, 1973; Reis *et al.*, 1975).

Recently, in preliminary experiments, we demonstrated using a TH cDNA probe that reserpine produced an increased accumulation of TH mRNA in rat adrenals and locus coeruleus (Mallet *et al.*, 1983). Similar results have also been obtained in the rat superior cervical ganglia (Black *et al.*, 1985). We now present the profiles of TH mRNA and enzyme changes after reserpine injection and discuss their possible significance to the mechanism of TH induction. To perform this study we developed a quantitative and sensitive Northern blot procedure to assay TH mRNA from about a minimum of 600 catecholaminergic cells of the brain.

Results

Analysis of TH mRNA species in various tissues

In a first series of experiments, poly(A)$^+$ RNA was purified from PC12 cells, locus coeruleus, substantia nigra and rat

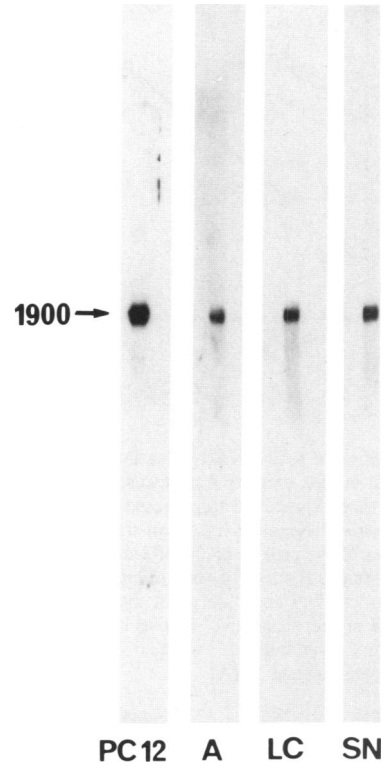

Fig. 1. Northern blot analysis of TH mRNA from various tissues: poly(A)$^+$ RNAs were denatured and separated by electrophoresis in a 1% denaturing gel. After migration, poly(A)$^+$ RNAs were transferred to nitrocellulose, baked under vacuum and hybridized with the cDNA pTH-1 probe. The filter was exposed to autoradiography for 24 h with an intensification screen at −70°C. A (adrenals): 15 μg; LC (locus coeruleus): 10 μg; SN (substantia nigra): 5 μg, were compared with control PC12 cells TH mRNA (0.5 μg).

adrenals. The analysis of TH mRNA in these tissues was performed by Northern blotting (Figure 1). In all structures, the TH cDNA probe hybridized to a single species and its size was identical to that previously reported for TH mRNA from PC12 cells (Lamouroux *et al.*, 1982; Chikaraishi *et al.*, 1983).

Development of a sensitive assay for TH mRNA using a small amount of tissue

To analyse pharmacological modulations of TH gene expression, a sensitive and quantitative method for detection of TH mRNA from milligram amounts of tissue was developed.

Total RNA was extracted from frozen (2−40 mg) samples.

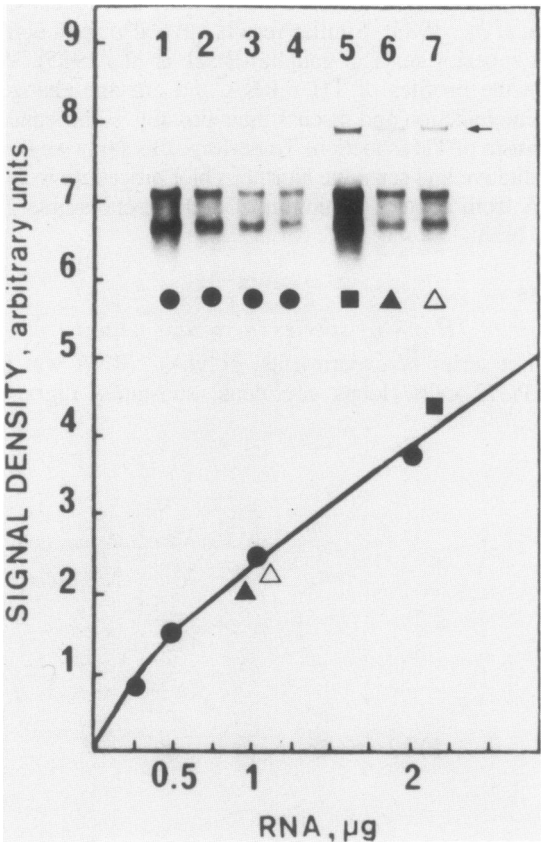

Fig. 2. Quantification of total RNA by densitometric scanning. An aliquot of 1/5 of the total nucleic extracts from locus coeruleus and substantia nigra and 1/10 of one adrenal were electrophoresed in agarose gels and stained with ethidium bromide. Negative images of u.v.-transilluminated gels were scanned. The area of the peak over the 18S and 28S ribosomal bands were compared with a standard curve of serial dilutions of liver RNA: **lanes 1, 2, 3, 4** represent 2, 1, 0.5 and 0.25 µg of total liver RNA, respectively. **Lanes 5, 6** and **7** are representative samples of adrenals, locus coeruleus and substantia nigra. The high mol. wt band in **lanes 5, 6, 7** (arrow) corresponds to contaminating DNA.

The addition of dextran T40 during the extraction procedure substantially increased the yield of RNA recovered. The total amount of RNA was quantified after migration on agarose gel by comparison of the level of 18S and 28S rRNA in the sample with that of known amounts of liver RNA, as described in Materials and methods. Figure 2 shows that a linear relationship exists between the amount of RNA and the signal density over the 18S and 28S ribosomal bands. In addition to the bands corresponding to 18S and 28S RNA, Figure 1 also reveals in most of the lanes a band corresponding to high mol. wt DNA. Less than 10% variation in the amount of RNA was obtained in the various extractions (Table I).

Total RNA from all drug-treated samples was not significantly different from the amount of total RNA in untreated samples: 5.0 (±0.7); 5.7 (±0.4); 34 (±4) µg for locus coeruleus, substantia nigra and adrenals, respectively.

TH mRNA was analysed by the Northern blot technique. The sensitivity of the method was greatly improved by the use of minigels for electrophoresis. The labelling of the probes by the random priming method led to specific activities that were 2- to 4-fold higher than labelling by nick translation. Dilutions of standards PC12 RNA were included on each gel and we only considered the TH mRNA values that were within the range of the standards. This method allowed reliable determination of tissue content of TH mRNA after correction for the amount of RNA in the structure (Figure 5). The same batch of PC12 RNA was used for the whole series of experiments to eliminate possible variations of TH mRNA levels in the cultured cells. The radioactivity corresponding to TH mRNA varied linearly with applied PC12 RNA for <120 ng, as shown in Figure 3. At higher RNA concentrations the relationship deviates from linearity because the [^{32}P]cDNA probe is no longer in excess and because of saturation of the film emulsion. Less than 30 ng of PC12 RNA is sufficient to reveal TH mRNA hybridizing bands and, as shown in the example of Figure 4, a band can be detected with only 1 µg of locus coeruleus RNA.

Time course changes of TH mRNA and enzyme activity after reserpine treatment

Rats were injected with a single dose of reserpine and changes in mRNA and enzyme activity were analysed at various times in locus coeruleus, substantia nigra and adrenals (Figure 5). Two dilutions of RNA derived from each tissue were used to compare levels of TH mRNA between injected and control rats. These four samples were analysed on the same gel and hybridized on the same filter. Less than 10% variation was obtained in the analysis of TH mRNA levels between the two dilutions and the relative change of TH mRNA for each experiment was the average of the two values. In locus coeruleus TH mRNA levels rose sharply after drug treatment and reached a peak corresponding to 450% of control values after 2 days. The maximum enzyme activity, however, was reached at day 4 with a value correspond-

Table I. Yield of RNA and activity of TH from various rat tissues

	LC[a]	SN	A
Wet weight (mg)	4.9 ± 0.6 (15)	5.8 ± 0.5 (15)	29.0 ± 5 (15)
RNA/structure[b] (µg)	4.3 ± 0.6 (15)	5.2 ± 0.4 (15)	60.7 ± 8 (15)
TH activity nmol DOPA/10 min/structure	0.68 ± 0.03 (10)	2.79 ± 0.19 (10)	26.97 ± 1.83 (10)

[a]The different values are tissues from one animal: i.e. both locus coeruli (LC), substantia nigrae (SN) and adrenal glands (A), treated as described in Materials and methods.
[b]Quantification of total RNA by densitometric scanning of the negative image of u.v.-transilluminated gel after ethidium bromide staining.
The values represent the mean ± SEM. The number of experiments is given in parentheses.

ing to only 260%. The rise of both TH mRNA and enzyme activity was still marked at day 8 and returned to control values after 18 days. The drug induced similar changes in adrenals, a maximum 4.1-fold increase in TH mRNA was also observed at day 2 but the decrease was sharper and after 4 days the TH mRNA level was only 140% that of control. The maximal level of enzyme activity also occurred at day 2 and slowly returned to control values at day 8. In contrast, no significant increase in TH mRNA level and activity was observed in substantia nigra.

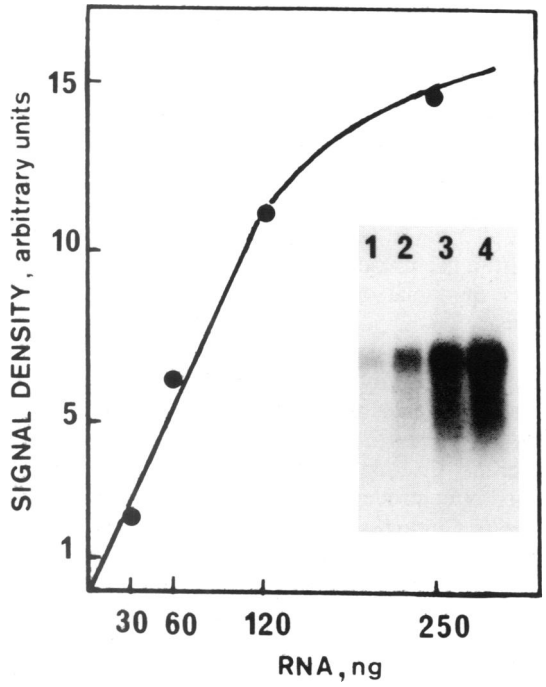

Fig. 3. Assay of TH mRNA by densitometric scanning. Serial dilutions of total RNA extracts from PC12 cells were electrophoresed after denaturation in a formaldehyde agarose gel. RNAs were blotted and hybridized with the cDNA pTH-1 probe. The resultant autoradiogram was scanned and the height of the peaks were plotted versus the amount (ng) of PC12 cell RNA.

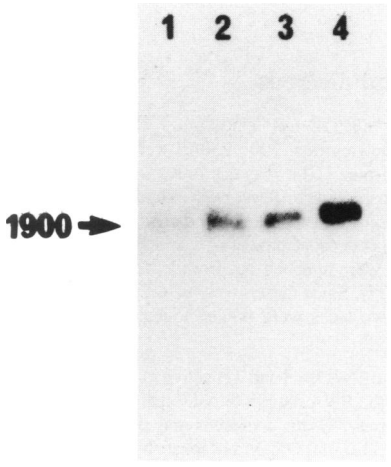

Fig. 4. Analysis of TH mRNA from total locus coeruleus RNA extract. Total RNA was extracted from locus coeruleus of one rat, electrophoresed, blotted and hybridized with TH cDNA probe (pTH-1). **Lanes 1** and **2** corresponded to 1 μg and 2 μg of RNA (1/5 and 2/5 of the total RNA of one locus coeruleus) of control animal: **lanes 3** and **4** correspond to 1 μg and 2 μg of a reserpine-treated animal.

Discussion

Here we have analysed in rat adrenals, locus coeruleus and substantia nigra the time course of changes of both TH mRNA and enzyme activity following the injection of a single dose of reserpine. This drug which interferes with the transport of catecholamines into the storage vesicles (Bianchi and Takimoto, 1984) induces a long lasting increase in TH activity and, therefore, provides a convenient model to study the mechanism of induction of this enzyme.

Most biochemical studies of catecholaminergic cells have been performed on the peripheral nervous system and, more particularly, on the adrenal medulla where they constitute the abundant population of chromaffin cells responsible for the secretion of noradrenaline and adrenaline. According to Nordmann (1985) each adrenal gland contains $\sim 2.88 \times 10^5$ chromaffin cells. In contrast, catecholaminergic cells of the central nervous system

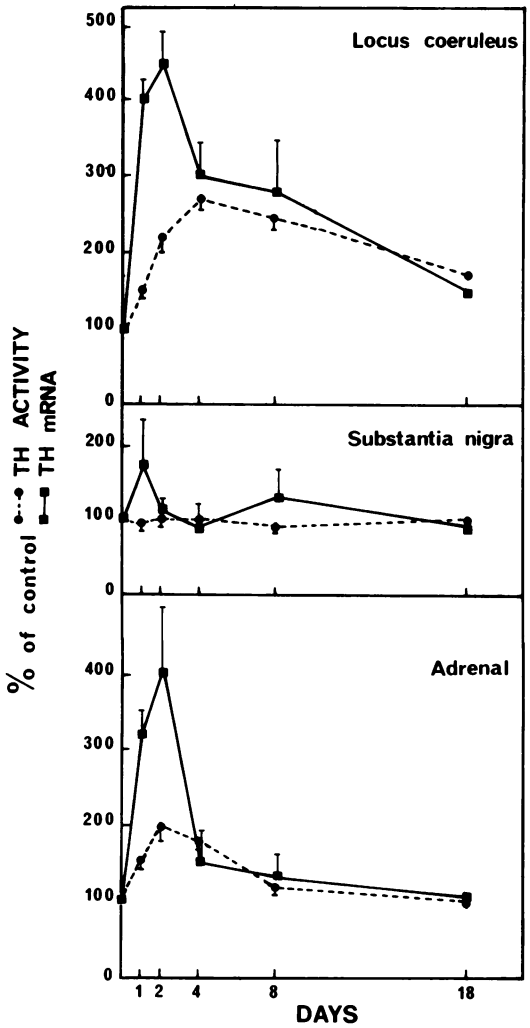

Fig. 5. Time course of changes in TH mRNA and TH activity in the locus coeruleus, substantia nigra and adrenals of rats at various days following administration of one single dose of reserpine (10 mg/kg, s.c.). The results are expressed as a percentage of the appropriate vehicle injected control rats which were sacrificed at the same date. Results of TH activity and TH mRNA are means ± SEM of 3–5 independant experiments. Control values were 55.7 (± 10.2) ng of equivalent PC12 RNA per locus coeruleus, 220 (± 19) ng per substantia nigra and 562 (± 108) per pair of adrenals; ng equivalent PC12 RNA correspond to the quantity of PC12 cell RNA that gives the same TH mRNA signal as the RNA of the structure analysed. At day 18 only one experiment was performed.

are found in small nuclei, mostly in the brain stem and mesencephalon. Substantia nigra and locus coeruleus constitute the most prominent dopaminergic and noradrenergic areas, respectively. In the rat, the locus coeruleus has been estimated to contain ~3000 cells that send projections to nearly every principal brain region. The substantia nigra is responsible for the dense innervation of the striatum (for review, see Moore and Bloom, 1978, 1979).

In a first set of experiments we have analysed TH mRNA in these various structures; poly(A)$^+$ was purified from 50 rats and a Northern blot analysis with the TH cDNA probe revealed only a single band of the same size in adrenals, locus coeruleus and substantia nigra (Figure 1). No cross-reactivity was observed with dopamine-β-hydroxylase in adrenal medulla and locus coeruleus although these two enzymes have been hypothesized to share sequence homology (Joh *et al.*, 1983). We then developed a sensitive method to analyse the time course of TH induction following reserpine treatment that allowed us to assay both TH mRNA and enzyme activity for a single rat. The sensitivity and reproducibility of this method was greatly improved by, in particular, the use of dextran T40 and electrophoresis on minigels. Some RNA preparations still contained small amounts of DNA. However, this contamination, which would have perturbed a dot-blot analysis, was not a problem for this method. The sensitivity of the technique, clearly illustrated in Figure 4, indicates that it is possible to detect TH mRNA in samples corresponding to ~600 noradrenergic cells of locus coeruleus. Many tissues can be processed simultaneously and this approach is practical for routine pharmacological tests.

Interestingly, from data in Table I and Figure 5 we can calculate that the 3000 catecholaminergic cells of a rat locus coeruleus contain about the same amount of TH mRNA as 57 000 chromaffin cells, indicating that a locus coeruleus cell contains ~20 times more TH mRNA than one chromaffin cell. This ratio is probably even higher for substantia nigra. The TH mRNA level represents a much higher percentage of total mRNA in catecholaminergic cells of the brain than in adrenals. Furthermore, locus coeruleus and substantia nigra contain approximately four times more TH mRNA per unit of enzyme activity than adrenals. These results can easily be rationalized by considering that somata of catecholaminergic brain cells have to synthesize large amounts of enzyme protein which are transported to the terminal areas. A high level of mRNA is necessary to sustain the great amount of enzyme activity throughout the terminal field. In contrast, in the adrenal medulla where the enzyme is not transported, both the amount of TH mRNA per cell and the ratio of TH mRNA to enzyme activity are lower.

The time course analysis shown in Figure 5 reveals that the increase in enzyme activity, elicited by reserpine in adrenals and locus coeruleus, is preceded by a sharp increase in TH mRNA that reaches a maximum level 2 days after the drug injection. These results as well as those obtained from actinomycin D experiments (Mueller *et al.*, 1969) suggest that the increase in enzyme activity reflects an enhanced transcription of the TH gene. No effect was observed in substantia nigra in agreement with the initial work of Reis *et al.* (1974). These results confirm and extend our previous finding that TH mRNA levels are increased in locus coeruleus and adrenals 4 days after reserpine treatment (Mallet *et al.*, 1983). The effect was, however, less pronounced and the most probable explanation may be related to differences in the method used: (i) in these initial experiments, tissues were pooled from several animals before analysis and it was therefore not possible to eliminate those animals in which the enzyme induction was not effective; (ii) the dot hybridization method which was used gave a higher background and therefore diminished the relative increase of TH mRNA levels.

Surprisingly, the present study revealed that both in the adrenals and locus coeruleus the maximum relative increase of TH mRNA is much higher than that of enzyme activity. Also, TH mRNA levels decrease sharply after day 2 and it seems that all the available pool of TH mRNA has not been processed efficiently to increase enzyme activity. The profiles of TH mRNA and activity declines are however quite different in adrenals and locus coeruleus. Clearly, reserpine elicits a much longer lasting effect in the brain nuclei than in the peripheral catecholaminergic cells. In the adrenals the TH mRNA level has almost returned to its initial value at day 4 whereas it is still 3-fold higher in the locus coeruleus. In fact, in this latter structure after day 4, both TH mRNA and enzyme levels decline slowly and the effect of the drug is still significant at day 18 confirming earlier TH activity studied by Zigmond (1979). At this stage, it is attractive to speculate that the difference in the amplitude of effects that are observed, after day 4, between locus coeruleus and adrenal results from a difference in the stability of the TH mRNA. Run on experiments on isolated nuclei should allow us to test this hypothesis.

Little is known about the sequence of events linking the increase in neural activity with that of TH mRNA. Various studies performed on adrenals and superior cervical ganglia indicate that acetylcholine is the first messenger of trans-synaptic induction. In the adrenal the role played by cAMP as a putative second messenger is controversial (Costa *et al.*, 1975; Thoenen and Otton, 1976; Guidotti and Costa, 1977). However the extensive data of Thoenen *et al.* (1979) suggest that cyclic nucleotides can be excluded as second messenger. Experiments with cultures of dissociated superior cervical ganglion neurons from newborn animals revealed that depolarisation *per se* increased TH activity (Hefti *et al.*, 1982), and recently using similar cultures we have shown that depolarisation with K^+ induced a 10-fold increase in TH mRNA (Raynaud *et al.*, in preparation). Changes in membrane electrical activity have also been found to regulate the level of acetylcholine receptor α-subunit mRNA (Klarsfeld and Changeux, 1985). How external influences induce long-lasting changes in neuronal properties has only begun to be explored.

Materials and methods

Reserpine treatment and dissection

Male Sprague-Dawley rats (200–250 g) were housed and treated with a single injection of reserpine (10 mg/kg) as previously described (Mallet *et al.*, 1983) except that either reserpine (Serva) or vehicle injections were administrated s.c.

Tissue dissection was as previously reported (Mallet *et al.*, 1983): adrenal glands were dissected and decapsulated at 4°C and rapidly frozen. Locus coeruleus and substantia nigra were punched out from frozen 500-μm thick brain serial slices (Buda *et al.*, 1975). Since these two structures have a bilateral localization, punches from the two sides were pooled together.

Isolation of RNA

As both the TH mRNA level and TH activity were determined in the same sample, the first step of the RNA extraction was performed with a buffer that preserved TH enzyme activity. Locus coeruleus and substantia nigra from each rat were homogenised in 50 μl of 0.002 M phosphate buffer pH 6.0 containing 0.2% Triton X-100 (Sigma). 10 μl was saved for enzymatic activity assay and 40 μl was used for total RNA preparation. RNA was isolated by homogenisation in 200 μl of buffer (0.2 M Tris pH 8.8, 25 mM EDTA, 0.1 M LiCl, 1% SDS), 5 μg/ml of dextran T40 (Pharmacia) and extracted twice with two volumes of phenol:chloroform:isoamyl-alcohol (25:24:1). RNA was purified by selective LiCl precipitation followed by precipitation by 70% ethanol (Lomedico and Saunders, 1976). Total RNA were resuspended in 5 μl of water, 1 μl used for total RNA quan-

tification and 4 μl used for mRNA analysis. One adrenal was treated as described above except that a larger volume of homogenisation buffer (100 μl) was used and a 20 μl aliquot was saved for the TH enzymatic assay. In some experiments, poly(A)$^+$ RNA was selected by an oligo(dT)-cellulose (PL Biochemicals, Type 7) chromatography (Aviv and Leder, 1972) using binding buffer (0.5 M NaCl, 0.01 M Tris-HCl, pH 7.5, 0.5% SDS) and elution buffer (0.01 M Tris-HCl, pH 7.5, 0.5% SDS). RNA fractions were precipitated in 70% ethanol and stored at −70°C. The integrity of the poly(A)$^+$ RNA preparations was checked by their ability to be efficiently translated in a reticulocyte lysate (Merrick, 1983).

Preparation of ^{32}P-labeled TH cDNA probe

All experiments were performed with the 742-bp *PstI* insert from pTH-1 cDNA clone (Lamouroux *et al.*, 1982). This fragment which is completely included in the coding region of the corresponding TH mRNA (Grima *et al.*, 1985) was purified after agarose gel elecrophoresis by absorption onto a NA 45 ion-exchange membrane (Schleicher and Schull, FRG) according to Dretzen *et al.* (1981). This fragment was labeled either by nick translation (Rigby *et al.*, 1977) using a commercial kit (Amersham) or by random priming (Feinberg and Vogelstein, 1983) with synthetic oligonucleotides (hexadeoxyribonucleotides no. 2166 PL Biochemicals Inc., USA). Specific activities of ~2 × 10^8 c.p.m. and 5−8 × 10^8 c.p.m., respectively were obtained. Both methods produced probes of an average size of 500 bp. Before hybridization they were denatured by boiling for 10 min.

Northern blot preparation

Electrophoresis of RNA was performed as described by Thomas (1980). RNA was denatured in 50% formamide, 6% formaldehyde buffer for 15 min at 65°C and electrophoresed in a 1% agarose gel containing 6% formaldehyde. The RNA were transferred to nitrocellulose membranes (BA 85 Schleicher and Schull, FRG) by a capillary blot procedure using 20 × SSC buffer. The membrane was dried and baked at 80°C under vacuum for 2 h. The transfer of RNA from the gel to the membrane filter was controlled in each case by ethidium bromide staining of the gel after extensive washings. For these experiments, we used a minigel apparatus of 10 cm in length (Minnie submarine, Hoefler Scientific Instruments, USA).

Hybridization and washings

Each blot was incubated for 5−7 h with 5 ml of pre-hybridization buffer containing 50% formamide, 5 × SSC, 50 mM Na phosphate pH 6.5, 0.02% bovine serum albumin (BSA), 0.02% polyvinyl pyrrolidone (PVP), 0.02% Ficoll, 250 μg/ml salmon DNA, 10 μg/ml poly(A) and 10% dextran sulfate at 42°C. Hybridization was performed for 18 h at 42°C in the same solution except that 50 μg/ml salmon DNA and 10^5 c.p.m./cm^2 of ^{32}P-labeled probe were added to the freshly made up hybridization buffer. The membrane was then washed twice with 100 ml of 2 × SSC, 0.1% SDS, once with 0.3 × SSC, 0.1% SDS and once with 0.1 × SSC, 0.1% SDS for 20 min at 65°C and then subjected to autoradiography (Fuji X-ray film) using an intensification screen (Dupont Cronex).

Assay of total RNA and TH mRNA

Two-fold serial dilutions of total RNA were electrophoresed in 0.8% agarose gel and stained with ethidium bromide at 1 μg/ml. The negative image of u.v.-transilluminated photographs of the gels were densitometrically scanned. The areas of the peaks over the 28S and 18S ribosomal bands were compared with standard curves of serial dilutions of liver RNA of known concentration run on the same gel (Eiden *et al.*, 1984).

TH mRNA was assayed by densitometric scanning of the autoradiograms. Serial dilutions of total RNA from PC12 cells were applied to each agarose gel, electrophoresed, blotted and hybridized to cDNA-TH. Resultant autoradiograms were scanned and the signal density was plotted as a standard curve. We selected exposure times of the film that produced a linear response between mRNA concentration and the intensities of the bands.

Tyrosine hydroxylase assay

TH activity in the brain was measured *in vitro* by a modification of the method of Coyle (1972) as follows. The aliquot of the homogenate saved for TH assay was diluted in the same buffer to a final volume of 60 μl for the locus coeruleus, substantia nigra and 400 μl for one adrenal. After centrifugation at 10 000 *g* for 15 min at 4°C, a 50 μl aliquot of the supernatant was transferred to 5 ml glass tubes. The reaction was initiated by addition of 50 μl of a mixture containing 10 μl of 1 M potassium phosphate buffer, pH 5.5, 5 μl of 10 mM NADPH (Boehringer Mannheim), 10 μl of 10 mM d,l-6-methyl-5,6,7,8-tetrahydropterine hydrochloride (Sigma), 3000 units of catalase (Boehringer Mannheim), 5 μl of 20 mM $FeSO_4$, and 10 μl of 0.4 mM cold tyrosine mixed with 2 × 10^5 c.p.m. 1-3,5-H tyrosine. The samples were incubated for 10 min at 37°C and the reaction was stopped by addition of 50 μl of 1 M perchloric acid. The mixture was passed over a 2 cm 50 W X 4 (200−400 mesh) Dowex column in a micropipette which was subsequently washed with 0.6 ml of water. The total effluent was collected in a plastic vial and the radioactivity counted by liquid scintillation.

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