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EVIDENCE FOR REGULATION OF TYROSINE HYDROXYLASE mRNA TRANSLATION BY STRESS IN RAT ADRENAL MEDULLA

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Abstract

Long-term stress leads to induction of tyrosine hydroxylase (TH) protein and enzymatic activity in the adrenal medulla. This adaptive response is necessary to maintain the catecholamine biosynthetic capacity of adrenal chromaffin cells during periods of sustained catecholamine secretion. In this report we demonstrate that when rats are subjected to short-term stress, TH mRNA is induced for at least 24 hr, but TH protein and TH activity (assayed under Vmax conditions) are not increased. In contrast, adrenal TH mRNA, TH protein and TH activity are induced in rats subjected to long-term stress. Using sucrose gradient fractionation, we show that the lack of induction of TH protein after one type of short-term stressor, a single 2 hr immobilization stress is associated with a decrease in the percentage of TH mRNA molecules associated with polysomes. In contrast, after repeated immobilizations the polysome profile of TH mRNA is identical to that observed in control animals, even though TH mRNA is induced 2–3 fold. These results are consistent with the hypothesis that even though TH mRNA is induced by short-term stressors, mechanisms that control TH mRNA translation must also be appropriately regulated for TH protein to be induced.

Keywords

tyrosine hydroxylase; adrenal medulla; stress; mRNA translational regulation

1. Introduction

An animal's response to stress is essential to its survival. It is required for the animal to compete successfully for food or mates, to flee from dangerous circumstances or to adapt to new activities or unfamiliar situations. However, these short-term beneficial responses can become pathological, if the stressful stimulus is excessive or prolonged (McEwen, 1998; McEwen and Stellar, 1993; Seeman et al., 1997). Hence, it is important to understand the mechanisms by which short-term responses to stress develop into long-term contributors to chronic pathological disorders. Many of these mechanisms involve changes in gene expression (Sabban and Kvetnansky, 2001).

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Some of the primary mediators of the acute stress response are the catecholamines, particularly norepinephrine and epinephrine. Tyrosine hydroxylase (TH) catalyzes the rate-limiting step in catecholamine biosynthesis; hence, it is of prime importance in maintaining the levels of these important neurotransmitters and hormones. TH is regulated by both short-term and long-term mechanisms. During acute stress, pre-existing TH molecules are activated by phosphorylation of serine sites on the N-terminus of the enzyme, leading to an increased affinity for the limiting cofactor, tetrahydrobiopterin (Kumer and Vrana, 1996). This activation occurs rapidly and promotes increased catecholamine biosynthesis immediately after stress-induced nerve stimulation.

During prolonged or repeated stress, TH protein is induced slowly over 12–24 hr. The mechanisms responsible for this induction in adrenal medulla have been partially elucidated (see Sabban and Kvetnansky, 2001; Sabban et al., 1998 for reviews). The prevailing model postulates that stress activates signaling pathways that stimulate TH gene transcription, resulting in induction of TH mRNA and TH protein. However, there are problems with this simple model. First of all, induction of TH mRNA does not always lead to induction of TH protein (Nankova et al., 1994; Piech-Dumas et al., 1999; Yoshimura et al., 2004). Secondly, even though most stressors stimulate TH gene transcription rate, the mechanisms responsible for this stimulation appear to be different depending on whether the stress is acute or chronic (Nankova et al., 2000; Nankova et al., 1999; Osterhout et al., 1997; Sun et al., 2003). Thirdly, there are a number of examples in which there is a lack of correlation between stress-induced increases in TH gene transcription rate and TH mRNA levels, providing evidence for regulation of TH mRNA stability (Alterio et al., 2001; Chang et al., 2000; Czyzyk-Krzeska et al., 1994a; Czyzyk-Krzeska et al., 1994b; Sun et al., 2004). Finally, there is increasing evidence that the response of the TH gene is dependent on the type of stressor and the tissue being investigated (Osterhout et al., 2005; Rusnak et al., 1998; Rusnak et al., 2001; Sun et al., 2004). These results suggest a more complex model, in which both transcriptional and post-transcriptional mechanisms participate in the stress-mediated induction of TH, and in which the type of stressor and the duration of the stress determine the mechanisms that control expression of the gene.

One example of this complex regulation is that elicited by immobilization stress. Repeated immobilization stress leads to a prolonged induction of TH protein in adrenal medulla (Kvetnansky et al., 1996; Nankova et al., 1994). This induction is associated with induction of TH mRNA and appears to be due to a sustained activation of TH gene transcription rate that occurs after 2–3 repeated immobilizations (Kvetnansky et al., 1996; McMahon et al., 1992; Nankova et al., 1994; Nankova et al., 2000; Nankova et al., 1999; Osterhout et al., 2005; Osterhout et al., 1997). In contrast, a single immobilization leads to a dramatic induction of TH mRNA that persists for at least 12 hr, but does not elicit a significant induction of TH protein or TH activity (McMahon et al., 1992; Nankova et al., 1994; Osterhout et al., 2005). In the present report we test whether two other short-term stressors elicit significant sustained increases in TH mRNA without concomitant increases in TH protein or TH activity. We also test whether this lack of induction of TH protein in the presence of induced TH mRNA in response to a single immobilization is due to a decrease in the percentage of TH mRNA molecules bound to polysomes. Our results support the hypothesis that a single episode of short-term stress leads to a decrease in TH mRNA template utilization for translation; whereas long-term or repeated stress regulates mechanisms that enhance TH mRNA template utilization, leading to induction of TH protein.

2. Results

Effect of single or repeated immobilization stress on adrenal TH expression

Rats were subjected either once or repeatedly for 7 days to a 2 hr immobilization stress. Adrenal glands were removed under anesthesia at 2 hr or 24 hr after the end of the final immobilization; TH activity, TH protein and TH mRNA levels were measured in these adrenals (Figure 1). Both single and repeated immobilization stress elicited large increases (5–7 fold) in TH mRNA levels 2 hr after the final stress and 2–3 fold increases at the 24 hr time point. In rats immobilized repeatedly for 7 days, adrenal TH activity increased 2–3 fold at both 2 hr and 24 hr after the final stress (Figure 1D). Similarly, significant increases in TH protein levels were observed at 2 and 24 hr after repeated immobilization stress. The slightly larger stress-induced increases in TH activity compared to the increases observed for TH protein might be due to activation of the enzyme. In contrast, neither TH protein levels, nor TH activity increased after a single immobilization stress (Figure 1C). These results essentially agreed with those reported in earlier studies (Nankova et al., 1994; Sun et al., 2004). In order to determine whether this lack of induction of TH protein and TH activity (even when TH mRNA was induced by at least 2-fold for 24 hr) was a unique response to a single immobilization stress, we tested the effects of other single episode or short-term stressors on adrenal TH mRNA, TH protein and TH activity.

Effect of 2-deoxyglucose (2-DG) injections on adrenal TH expression

Rats were injected either once or repeatedly (one injection each day for 7 days) with 0.5 mg/kg 2-DG. Adrenal glands were removed at 5 hr or 24 hr after the final injection. TH mRNA levels increased ~5-fold when measured 5 hr after a single injection of the glucose analog and remained elevated (2–3 fold) at 24 hr (Figure 2). In contrast, TH protein levels did not change significantly at either 5 hr or 24 hr after this single 2-DG injection, and TH activity increased only slightly at the 5 hr point. After rats were injected 7 times with 2-DG, TH mRNA levels increased 3–5-fold, when measured at either 5 hr or 24 hr after the final injection (Figure 2). TH activity and TH protein levels increased 2–4 fold after 7 daily 2-DG injections. As observed for immobilization stress, TH activity increased to a greater extent than TH protein after repeated 2-DG injections; this greater increase in enzyme activity might be due to enzyme activation, but this possibility was not explored in this study.

Effect of short-term cold exposure on adrenal TH expression

When rats were subjected to cold exposure for 5 hr, adrenal TH mRNA levels increased by ~2-fold at both 9 hr and 24 hr after the initiation of the stress (Figure 3). In contrast, neither TH activity, nor TH protein increased significantly at either of these two time points. Previous studies from numerous laboratories have shown that long-term exposure to cold elicits 2–3 fold increases in both TH mRNA and TH activity in the adrenal (Baruchin et al., 1990; Bhatnagar et al., 1995; Stachowiak et al., 1986; Tank et al., 1985).

Polysomal profile of TH mRNA in rat adrenal medulla

To test whether the lack of induction of TH protein in response to a single episode of short-term stress was due to inefficient translation of the induced TH mRNA, we chose to use immobilization as a stressor and assayed the amount of TH mRNA present in polysomes in the adrenals of control and immobilized rats. Adrenal cytoplasmic extracts were subjected to sucrose gradient centrifugation to separate polysomal and nonpolysomal RNA. Total cytoplasmic RNA levels (estimated by continuous monitoring of the sucrose gradient effluent at 254 nm) were higher in the light fractions (top of the gradient which contains nonpolysomal mRNPs) than in the heavier fractions (bottom of the gradient which contains polysomal-bound RNA). A peak representing 80S ribosome particles is observed near the center of the gradient

(Figure 4A). This cytoplasmic RNA pattern agreed closely with that observed in previous studies using brain samples (Bagni et al., 2000; Krichevsky and Kosik, 2001). The polysomal distributions of different mRNAs were estimated by measuring their levels in different fractions isolated from the gradient using semiquantitative RT-PCR. Representative autoradiograms of these assays are presented in Figure 4B and quantitative plotting of the results are shown in Figures 4C and 4D.

In adrenals isolated from control, unhandled rats, TH mRNA levels were distributed throughout the gradient. Two broad peaks were discernable; one peak ranging between fractions 3–9 and a second peak ranging between fractions 12–16. This profile suggested that adrenal TH mRNA was localized in both nonpolysomal and polysomal compartments. This result was consistent with the hypothesis that only a portion of the total cellular concentration of TH mRNA was being actively translated under nonstressed conditions. To verify this conclusion, a number of control experiments were performed. (1) Adrenal cytoplasmic extracts were initially treated with EDTA prior to sucrose gradient centrifugation. The assembly of polysomes is dependent on divalent cations; hence, the addition of EDTA to the extracts would be expected to diminish the amount of TH mRNA in the polysomal fractions, if these fractions truly represented polysomal-bound TH mRNA. As seen in Figures 4B and 4C, EDTA treatment dramatically decreased the levels of TH mRNA localized in the fractions with the heaviest sucrose densities ($\Sigma \text{Fractions (F)}_{12-16} = 37\%$ for TH mRNA without EDTA treatment; whereas $\Sigma F_{12-16} = 4.8\%$ with EDTA treatment). This result lent support to the hypothesis that these fractions represented polysomal-bound TH mRNA. (2) Ferritin H mRNA levels were measured in the fractions from the same sucrose gradient used for measuring TH mRNA levels; ferritin H mRNA is not actively translated in the absence of iron. As expected, ferritin H mRNA was localized completely in the fractions of the gradient (fractions 2–7) with the lightest sucrose densities, suggesting that these fractions represented nonpolysomal-bound RNA. (3) Two other mRNAs that are selectively expressed in adrenal medullary cells were measured to identify fractions containing polysomal-bound RNA. As shown in Figures 4B and 4D, even though significant levels of dopamine beta-hydroxylase (DBH) and aromatic amino acid decarboxylase (AADC) mRNAs were observed throughout the gradient, the greatest concentrations of these mRNAs were isolated in fractions 12–16 ($\Sigma F_{12-16} = 50 - 60\%$). We also measured the polysome profile of the mRNA encoding the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase in these same sucrose gradients; its profile was essentially identical to that of AADC mRNA (data not shown). Based on these control experiments, it seemed reasonable to designate the amount of TH mRNA present in fractions 12–16 as an estimate representative of polysomal-bound TH mRNA.

Effect of immobilization stress on the polysomal profile of TH mRNA in rat adrenal medulla

Rats were subjected to a single 2 hr immobilization or seven daily 2 hr immobilizations. In preliminary studies, we found that the polysome profiles from control rats that were unhandled or from control rats that were handled for 7 days were similar. Hence, for these studies we used unhandled rats as controls. Cytoplasmic extracts were isolated from adrenal glands 24 hr after the final immobilization and fractionated using sucrose gradient centrifugation. TH mRNA levels were measured in fractions isolated from these gradients. Results from three separate experiments are shown in Figure 5. As observed previously, adrenal TH mRNA levels in control rats were distributed throughout the gradient with ~33% of the total TH mRNA pools found in fractions 12–16, suggesting that about one-third of TH mRNA molecules in the adrenal medulla were being actively translated on polysomes in control animals (Figure 5A). In contrast, when animals were subjected to a single immobilization stress, TH mRNA distribution on the sucrose gradients was shifted to the left. TH mRNA levels were induced ~2.2-fold in these experiments at this time point; however, only ~21% of cytoplasmic TH mRNA was bound to polysomes in fractions 12–16, suggesting that a smaller percentage of

the TH mRNA pool was actively translated in these adrenals compared to those isolated from control rats. When animals were subjected to repeated immobilizations over seven days, TH mRNA distribution on the sucrose gradients returned to that observed under control conditions (~28% of TH mRNA molecules were isolated in fractions 12–16), even though total TH mRNA levels were increased by ~2.5-fold (Figure 5B).

As a control, the distribution of DBH mRNA was also assayed in these same sucrose gradients (Figure 5C). In unstressed animals, more than 50% of DBH mRNA was isolated in the polysomal fractions. When animals were subjected to a single immobilization stress, DBH mRNA did not increase significantly, which is in agreement with a previous report (McMahon et al., 1992). There was a slight shift to the left of the DBH mRNA polysome profile after a single immobilization, but this shift was not as great as that observed for TH mRNA and occurred primarily within the section of the gradient encompassed by fractions 12–16. This slight shift to the left for DBH mRNA was also observed by Wong and Wang (1994) after reserpine administration. In that report and the present study, even though there was a slight shift of DBH mRNA to the left in the gradient profile, the amount of DBH mRNA isolated in the polysomal fractions (ΣF_{12-16}) was not significantly changed by either immobilization stress or reserpine treatment. In the present study, ~61% of DBH mRNA was bound to polysomes in adrenals isolated from immobilized rats.

3. Discussion

The mechanisms responsible for TH induction by stress in adrenal medulla have been extensively studied (Sabban and Kvetnansky, 2001; Sabban et al., 1998). Stress promotes release of both cholinergic and noncholinergic neurotransmitters from the splanchnic nerve. These neurotransmitters bind to cognate receptors on adrenal chromaffin cells, resulting in activation of signaling pathways that stimulate TH gene transcription rate. The available evidence supports a model in which a single, short-term episode of stress leads to TH gene stimulation via activation of transcription factors that interact with TH AP1 and TH CRE sites within the proximal TH gene promoter. If the gene is stimulated for at least 2 hr, TH mRNA is induced. Long-term or repeated stress leads to a more robust and sustained induction of TH mRNA lasting for many days, due to the induction of long-lived transcription factors that stimulate TH gene transcription rate for a prolonged period of time (Nankova et al., 2000; Nankova et al., 1999; Osterhout et al., 1997; Sun et al., 2004).

Even though it is clear that transcriptional mechanisms play a major role in mediating induction of TH during stress, there is evidence that post-transcriptional mechanisms also participate in this response. For instance, cold exposure elicits a rapid induction of adrenal TH mRNA that is maximal after 3–6 hr and is sustained for at least 72 hr; however, TH protein isn't induced until 24 hr after this continuous cold stress (Baruchin et al., 1990). Nankova et al (1994) have shown that TH mRNA is dramatically induced (~8-fold) for 12–24 hr after a single immobilization stress; however, TH protein is induced by less than 2-fold and TH activity is not significantly elevated at any time point after this single immobilization. More recently, our laboratory has shown that either short-term repeated injections over 3–4 hr or long-term repeated injections twice per day for 7 days with the muscarinic acetylcholine receptor agonist bethanechol elicits a 2–3 fold induction of adrenal TH mRNA that persists for at least 12–24 hr; yet TH protein and TH activity are not induced by these treatments (Piech-Dumas et al., 1999; Yoshimura et al., 2004). These studies indicate that some types of stimuli induce TH mRNA without a consequent induction of TH protein.

In this report we present evidence that three single episode, short-term stressors induce adrenal TH mRNA for a sustained period of time (at least 24 hr) without concomitant increases in TH protein or TH activity. In contrast, long-term or repeated stress leads to induction of both TH

mRNA and TH protein. These latter results are in good agreement with work from numerous laboratories showing that almost all forms of long-term stress are associated with induction of adrenal TH mRNA, TH protein and TH activity (Stachowiak et al., 1986; Tank et al., 1985; Thoenen, 1970; Baruchin et al., 1990; Bhatnagar et al., 1995; DeCristofaro and LaGamma, 1994; Fluharty et al., 1983; Gagner et al., 1985; Kvetnansky et al., 1971; Kvetnansky et al., 2003).

One explanation for these results is a kinetic argument; TH mRNA may not be induced for a sufficient period of time after a single short-term stress to elicit a measurable induction of TH protein, due to its long half-life. An alternative hypothesis is that even though short-term stress induces TH mRNA for at least 24 hr, post-transcriptional mechanisms that control TH mRNA translation are rate-limiting for TH protein synthesis. The studies in this report provide the first direct evidence supporting this latter hypothesis. Using sucrose gradient fractionation procedures, we show that under control conditions about 33% of adrenal TH mRNA is associated with polysomes. Presumably, this fraction of TH mRNA is being actively and efficiently translated. A number of control experiments were performed to assess the validity of this conclusion. The experiment in which the samples were pretreated with EDTA to disassemble the polysomes indicates clearly that fractions 12–16 contain polysomal mRNAs. However, it is possible that some of the polysomes disassemble during the cell fractionation and sucrose gradient centrifugation procedures. If so, then the amount of TH mRNA in fractions 12–16 may underrepresent that present in the intact cell. To test for this possibility, we have measured the polysome profiles of two other mRNAs that are highly expressed in adrenal medulla; those encoding AADC and DBH. Both these mRNAs localize primarily (50–60%) to fractions 12–16. It is possible that the remaining 40–50% of these mRNA molecules, which are isolated from fractions containing lower sucrose densities, represent mRNA in nontranslatable cellular pools that are involved either in the transport of newly-synthesized mRNAs to polysomes or in mRNA degradation. Alternatively, the AADC and DBH mRNAs in these low density fractions may represent an artifact of the experimental protocol. In either case, it is clear that a smaller amount of TH mRNA is present in polysomal fractions than that observed for either DBH or AADC mRNA ($33 \pm 3\%$ for TH mRNA compared to $52 \pm 3\%$ for DBH mRNA and $50 \pm 5\%$ for AADC mRNA [$p < .05$ for either DBH or AADC mRNA compared to TH mRNA]). Since TH, DBH and AADC mRNAs were measured in fractions from the same sucrose gradients, this difference in polysomal profiles cannot be attributed to differences in artifactual disassembly of polysomes during different isolations. The most likely explanation is that a greater percentage of TH mRNA molecules reside in non-polysomal and consequently inefficiently translated cellular pools under control conditions. This key finding suggests that a post-transcriptional mechanism that specifically regulates TH mRNA translation may be rate-limiting for TH protein synthesis, even in unstressed animals.

The most significant finding in this study is that after a single immobilization stress the polysomal distribution of TH mRNA is shifted to the less dense fractions of the sucrose gradient. This shift indicates that a lower percentage of TH mRNA molecules are apparently associated with polysomes and hence a lower percentage of these molecules are being efficiently translated after a single immobilization. The simplest interpretation of this finding is that short-term stress leads to induction of TH mRNA, but that mechanisms controlling its assembly into polysomes are rate-limiting for its translation. Hence, a large amount of the induced TH mRNA is not used for TH protein synthesis.

When rats are subjected to repeated immobilization over many days, the polysome distribution of TH mRNA is similar to that observed in control animals, even though TH mRNA is induced 2–3-fold. One interpretation of this result is that after repeated immobilization, the rate-limiting mechanisms that control the assembly of TH mRNA into polysomes are up-regulated, such that the induced pool of TH mRNA molecules is translated as efficiently as the pool of TH

mRNA present under control conditions. Based on this interpretation, it is not surprising that TH protein is significantly induced after repeated or long-term stress. Alternatively, short-term stress may modulate mechanisms that lead to inhibition of TH mRNA polysome assembly and long-term stress may alleviate these inhibitory mechanisms. More work is needed to differentiate between these and other possibilities and to identify the molecular mechanisms that mediate this apparent translational regulation.

From a physiological standpoint, it is essential that the activity of TH be expressed at an appropriate level, in order to maintain the capacity to efficiently synthesize sufficient catecholamines, particularly during periods of enhanced nerve stimulation and consequent enhanced catecholamine release. During long-term stress and the attendant sustained increase in catecholamine secretion, TH enzyme protein is induced for a prolonged period of time. This induction is mediated by increases in TH gene transcription rate and TH mRNA levels. In contrast, during a single episode of short-term stress, TH protein is not usually induced. Instead, pre-existing enzyme molecules are activated by phosphorylation mechanisms. Presumably, this activation is sufficient to compensate for the loss of catecholamines during short periods of enhanced secretion. Hence, even though TH mRNA is induced during short-term stress, our results suggest that its translation is minimized, since increased levels of enzyme protein are not required. However, as the stress is sustained over longer periods of time, appropriate translational mechanisms are induced and TH protein is synthesized. According to this model, translational regulation of induced TH mRNA may play a vital role in fine-tuning the capacity of the adrenal to synthesize catecholamines during periods of stress or other types of stimulation.

4. Experimental Procedures

Treatment of rats

Male Sprague-Dawley rats (175–250 gm) purchased from Charles-River Laboratories were used in this study. Immobilizations were performed as described by Kvetnansky et al. (1996). Briefly, the rats were placed on their stomachs on a metal board; each of their limbs was taped to the board and the head was restrained in a metal ring fixed to the board. For experiments involving a single immobilization, rats were immobilized for 2 hr in the morning; controls were unhandled. For repeated immobilization experiments, rats were immobilized for 2 hr in the morning of each day for 7 consecutive days; controls were handled gently for 1–2 min each day. Animals were euthanized either 2 hr or 24 hr after the end of the final 2 hr immobilization (4 hr or 26 hr after the initiation of the stress). Protocols for 2-deoxyglucose (2-DG) treatment and cold stress were based on work by Rusnak et al. (1998; 2001). 2-DG was dissolved in saline and was administered subcutaneously either once or once per day for 7 consecutive days at a dose of 0.5 mg/kg. Control rats were injected with the same volume (0.5 ml) of saline. Cold-exposed rats were placed in a cage (two animals per cage) with minimal bedding for 5 hr at 4°C. At the end of this cold exposure rats were removed from the cold room and maintained at room temperature in the same cage until adrenals were removed. Control animals were maintained at room temperature and housed 2 animals per cage. At the appropriate time after the stress, the rats were administered a lethal dose of sodium pentobarbital (150 mg/kg). Adrenal glands were removed while the animals were anesthetized prior to death and either used immediately or frozen on dry ice. All procedures and drug administrations were performed in accordance with the guidelines and approval of the University of Rochester Committee on Animal Resources.

Measurement of TH enzyme activity and TH protein

TH activity was assayed as described previously (Fossom et al., 1991b). Briefly, frozen adrenal glands were homogenized in 250 μ l of 30 mM potassium phosphate (pH 6.8), 50 mM NaF and

10 mM EDTA, and the homogenate was centrifuged at 15,000g for 10 min. TH activity was measured by a coupled decarboxylation assay under apparent V_{max} conditions, using 4 mM 6-methyl-5,6,7,8-tetrahydropterin as cofactor. Endogenous catecholamines and other small molecules that might interfere with the assay were removed by subjecting the adrenal supernatants to gel filtration using Sephadex G-50 columns, equilibrated with 30 mM potassium phosphate (pH 6.8), 10 mM NaF and 0.1 mM EDTA. A 50 μ l aliquot of the gel-filtered supernatant was used for the assay. Protein was measured by the method of Bradford (1976), using bovine serum albumin as a standard. Adrenal TH activity was expressed as nmol $^{14}\text{CO}_2$ formed per min per mg protein.

TH protein was measured using western analysis as described in previous publications (Fossom et al., 1991a; Piech-Dumas et al., 1999; Sterling and Tank, 2001). The same adrenal supernatants used for assaying TH activity were used for measuring TH protein. Briefly, adrenal supernatant protein (10 μ g) was loaded onto a 10% SDS-polyacrylamide gel. In addition, a known amount of purified rat pheochromocytoma TH protein was loaded onto a separate lane for each gel for normalization purposes. The samples were subjected to electrophoresis, transferred to nitrocellulose and immunoblotted using rabbit antiserum specific for TH (this antibody recognizes both phosphorylated and nonphosphorylated forms of TH). The antibody-TH complexes were detected using the Amersham ECL system and autoradiography as described by Piech-Dumas et al. (1999). The autoradiographic bands were quantitated by scanning the autoradiograms with a Hewlett-Packard ScanJet 4C scanner with a transparency adaptor along with computer-assisted imaging analysis using NIH Image software to calculate the density units. Care was taken to use only those density values that were within the linear range of the autoradiographic film. The density units for each TH protein band were normalized to the amount of total soluble protein loaded onto the gel for that sample and then divided by the density units for the known amount of purified TH protein loaded onto that gel. TH protein was expressed as the fold-increase over controls measured on the same blots.

Measurement of TH mRNA using semi-quantitative RT-PCR

Semi-quantitative RT-PCR assays were performed as previously described (Sun et al., 2003; 2004; Yoshimura et al., 2004). Adrenal RNA (0.8 μ g) was subjected to RT using random hexamer primers. Aliquots of the resulting single-stranded cDNA products were used along with the appropriate primers (see below) in the PCR to incorporate α - ^{32}P -dATP (0.25 μ Ci per reaction) into double-stranded products encoding 519 bp TH cDNA, 295 bp 28S cDNA, 200 bp ferritin H cDNA, 326 bp DBH cDNA or 518 bp AADC cDNA. Except for 28S rRNA (which is encoded by an intronless gene), the 5' and 3' primers (see below) used for the PCRs were selected such that they encoded regions of different exons separated by an intron; therefore, PCR products derived from genomic DNA contaminating the isolated RNA would produce a detectably larger PCR band. These bands were never observed. All RT-PCR assays were run under conditions of linearity with respect to both cycle number and RNA input.

Gene Specific Primers

The primers for the PCR amplification of TH, DBH, AADC, ferritin H and 28S cDNAs were selected using the rat cDNA sequences for these mRNAs in GenBank (NCI/Frederick Biomedical Supercomputing Center) and the program OligoR. The following gene specific primers were used (the sequences are written from 5' to 3'): TH mRNA, 5' primer encoding sequences 390 to 410 (ccccacctggagtattttgtg) and 3' primer complementary to sequences 888 to 908 (atcacggcgacagtagacc); 28S rRNA, 5' primer encoding sequences 1–22 (gtgaacagcagttgaacatggg) and 3' primer complementary to sequences 276 to 295 (aaccgcgacgtttccaag); ferritin H mRNA, 5' primer encoding sequences 211 to 231 (gagttgatgcctctacgtc) and 3' primer complementary to 393 to 411 (ccagtcacacggtcaggt);

DBH mRNA, 5' primer encoding sequences 866 to 887 (actccaagatgaaacctgacag) and 3' primer complementary to 1172 to 1192 (tgtctgtgcagtagccagtc); AADC mRNA, 5' primer encoding sequences 424 to 443 (gaagatgcttgagctgccag) and 3' primer complementary to 923 to 942 (gaatctgcaaactccacgcc); and internal standard TH gene promoter sequences, 5' primer encoding sequences -385 to -362 (ggagacatatctagaagccctctc) and 3' primer complementary to -181 to -161 (gtgggccagctcttggaatac).

Polysome profile analysis of adrenal mRNAs

Polysome profile analysis was performed as described by Krichevsky and Kosik (2001). Adrenal glands were removed under anesthesia and immediately homogenized in ice-cold low-salt lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 1 mM RNasin, 1 mM dithiothreitol, 0.3% Triton X-100, and 0.05 M sucrose). Nuclei and the majority of mitochondria were sedimented by centrifugation for 10 min at 10,000g at 4°C. The NaCl and MgCl₂ concentrations in the supernatants were adjusted to 170 and 13 mM, respectively. Linear sucrose gradients (15% – 45% w/w in 25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 5 mM MgCl₂) were prepared using a Hoefer Scientific Instruments (San Francisco, CA) gradient maker with a sublayer of 0.5 ml of 45% sucrose in the same buffer. Cytoplasmic extracts (1 ml) were overlaid onto 9 ml gradients and centrifuged at 36,000 rpm for 2 hr at 4°C in a SW40 rotor. Fractions (625 ul) were separately collected with continuous UV monitoring at 254 nm using an ISCO UA-6 detector. RNA in each fraction was precipitated with isopropanol, extracted with phenol-chloroform-isoamylalcohol (25:24:1) and precipitated again using ethanol; the precipitated RNA was collected by centrifugation. The RNA pellet was washed once with 70% ethanol, and then suspended in 15 ul DEPC-treated water. Five ul of this RNA solution were subjected to RT using random hexamer primers as described above. To control for differences in recovery of RNA from each fraction during the isolation procedure, a known amount (100 ng) of a standard control RNA was added to each fraction before RNA isolation. This standard RNA was obtained by *in vitro* transcription (using a kit from Ambion, Austin, TX) of TH genomic DNA promoter sequences and detected using specific primers (see above). For EDTA treatment, MgCl₂ concentration in the first supernatant was not adjusted; 10 mM EDTA was added to these supernatants and the supernatants were incubated on ice for 10 min before they were loaded onto the sucrose gradients. The amount of polysomal mRNA in each gradient was estimated by calculating the Σ Fractions (F)_{12–16}, which is equal to the amount of mRNA quantified in fractions 12 – 16 divided by the total amount of mRNA quantified in all 16 fractions and expressed as a percentage of the total mRNA isolated on the gradients.

Statistical analyses

The results were analyzed by one-way analysis of variance, using the computer program INSTAT. Comparisons between groups were made using the Student-Neumann-Kuels or Dunnett multiple comparisons test. Student's t-test was used, when only two groups were being compared. A level of $p < .05$ was considered statistically significant.

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Abbreviations

TH	tyrosine hydroxylase
AADC	aromatic amino acid decarboxylase

DBH

dopamine beta-hydroxylase

2-DG

2-deoxyglucose

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Figure 1. Effects of immobilization stress on adrenal TH gene expression

Rats were immobilized either once or repeatedly for 2 hr each day for 7 days. For the single immobilization experiments, controls were left unhandled. For repeated immobilization experiments, control animals were handled gently for 1–2 min each day. Adrenal glands were removed from anesthetized rats 2 hr or 24 hr after the end of the final immobilization. (A) TH mRNA and 28S rRNA levels were assayed using semiquantitative RT-PCR; panel A depicts autoradiograms of representative assays. For each sample the density of the TH mRNA signal was normalized to the density of the 28S rRNA signal. (B) TH protein was assayed using western analysis; panel B depicts representative western blots. (C) TH activity was measured under Vmax conditions, using 4 mM 6MPH₄. The bar graphs represent the data expressed as fold-increases over controls for TH mRNA, TH protein and TH activity measured in adrenals from the same animals. The results are derived from two separate experiments. The data represent the means $\pm$ SE from 4–5 rats. a: $p < .01$ compared to controls

Figure 2. Effects of 2-DG treatment on adrenal TH gene expression

Rats were injected either once or repeatedly (once per day) with 0.5 mg/kg 2-DG. Control animals were injected with saline. Adrenal glands were removed under anesthesia at 5 hr or 24 hr after the final injection. TH mRNA and 28S rRNA levels were measured using semiquantitative RT-PCR; a representative autoradiogram is shown on the left-hand side of the figure. TH protein was measured using western analysis; a representative western blot is shown on the left-hand side of the figure. TH activity was assayed under Vmax conditions. The data in the bar graph represent the means $\pm$ SE from 3–4 rats from a single experiment; this experiment was repeated twice with similar results.

a: $p < .01$ compared to controls

Figure 3. Effect of short-term cold exposure on adrenal TH gene expression

Rats were maintained at 4°C for 5 hr. Adrenals were removed under anesthesia either 9 hr or 24 hr after the initiation of the cold exposure. Control rats were maintained at room temperature. TH mRNA was measured using semiquantitative RT-PCR; a representative autoradiogram is shown on the left-hand side of the figure. TH protein was measured using western analysis; a representative autoradiogram is shown on the left-hand side of the figure. TH activity was assayed under Vmax conditions. The results are derived from two separate experiments. The data represent the means $\pm$ SE from 5 animals.

a: $p < .01$ compared to controls

Figure 4. Polyribosome profile analysis of rat adrenal mRNA

Rat adrenal cytoplasmic extracts were fractionated by sucrose gradient centrifugation. Fractions from each gradient were collected while continuously monitoring absorbance at 254 nm. (A) Absorbance profile of a sucrose gradient showing a typical polysomal RNA distribution. (B) Autoradiograms of semiquantitative RT-PCR analyses of gradient fractions using primers specific for TH, ferritin H, DBH and AADC mRNAs and for a synthetic internal standard RNA that was added in an equal amount to all fractions prior to RNA isolation. For EDTA treatment, cytoplasmic extracts were treated with 10 mM EDTA on ice for 10 min before sucrose gradient centrifugation. (C) and (D) Results of phosphorimager scanning of the RT-PCR autoradiogram; mRNA levels in each fraction were expressed as a percentage of total levels summed over all 16 fractions. The TH mRNA values in both panels C and D are derived from the same analysis and are presented in both panels for comparison with the profiles of the different mRNAs analyzed in C and D.

Figure 5. Effects of immobilization stress on adrenal TH mRNA and DBH mRNA polysome profiles (A) Rats were immobilized once for 2 hr. Controls were left unhandled. Adrenal glands were removed 24 hr after the immobilization stress. Cytoplasmic extracts were prepared and subjected to sucrose gradient centrifugation. TH mRNA in each fraction of the gradient was assayed by semiquantitative RT-PCR. The upper panel shows an autoradiogram depicting TH mRNA levels in each sucrose gradient fraction of representative gradients. The lower panel depicts the results of phosphorimager scanning analyses of the RT-PCR autoradiograms, expressed as a percentage of total TH mRNA signal summed over all 16 fractions. The data represent the means $\pm$ SE from 3 experiments. (B) Rats were immobilized repeatedly for 2 hr for 7 consecutive days. Adrenal glands were removed 24 hr after the last immobilization stress.

Upper panel depicts representative autoradiograms; the lower panel depicts the density scanning results, expressed as a percentage of the total TH mRNA signal. The data represent the means $\pm$ SE from 3 rats. (C) DBH mRNA levels were measured in the same fractions isolated from the sucrose gradients described under panel A. a: $p < .01$ compared to control values.