

Chronic Nicotine Treatment Leads to Induction of Tyrosine Hydroxylase in Locus Ceruleus Neurons: The Role of Transcriptional Activation

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ABSTRACT

Chronic nicotine treatment (two daily subcutaneous injections administered ~12 h apart for 14 days) is associated with long-term inductions of tyrosine hydroxylase (TH) protein and TH mRNA in locus ceruleus (LC) neurons. These increases persist for at least 3 days after the final nicotine injection in LC cell bodies and for at least 7 to 10 days in LC nerve terminal regions. We tested whether this long-term response is due to sustained stimulation of TH gene transcription rate. A semi-quantitative reverse transcription-polymerase chain reaction assay was developed to assess changes in the levels of TH RNA primary transcripts; these changes are an indirect measurement of changes in TH gene transcription rate. TH RNA primary transcript levels increase rapidly in the LC after a single nicotine administration and return to basal levels by 24 h. A

similar rapid and transient induction of LC TH RNA primary transcripts occurs after chronic nicotine administration. In contrast, TH RNA primary transcript levels remain elevated for a sustained period of time (at least 1 day) in the adrenal medulla after chronic nicotine administration. Similar rapid, but transient changes in LC TH RNA primary transcript levels are observed after repeated immobilization stress. These results suggest that TH gene transcription rate in the LC is stimulated rapidly after each nicotine injection; however, in contrast to the adrenal medulla, there is no sustained transcriptional response elicited by chronic nicotine treatment or repeated immobilization stress in the LC, suggesting that post-transcriptional mechanisms may also play a role in these long-term responses.

Many of the addictive properties of drugs of abuse, like nicotine, are at least partially mediated by catecholaminergic neurons in the brain (Nestler et al., 1999; Dani and De Biasi, 2001; Dani et al., 2001). Chronic nicotine treatment elicits long-lasting effects on these neurons; many of which are due to the ability of the drug to modulate gene expression (Harlan and Garcia, 1998; Dani and De Biasi, 2001). One of the genes that is up-regulated by nicotine encodes tyrosine hydroxylase (TH), the enzyme that catalyzes the rate-limiting step in catecholamine biosynthesis. TH is induced by stimuli that evoke the release of catecholamines for prolonged periods of time. This induction represents a compensatory response, such that catecholamine biosynthetic capacity is enhanced to maintain the neuronal stores of these neurotransmitters even during periods of sustained secretion, as may occur during chronic nicotine administration.

A number of studies have shown that chronic nicotine

administration leads to induction of TH in locus ceruleus (LC) neurons. Smith et al. (1991) were the first to show that chronic nicotine treatment over a 3-week period leads to an increase in TH activity in the LC, as well as in the brain regions innervated by LC neurons, like the hippocampus, cerebellum, and frontal cortex. In a subsequent report, Mitchell et al. (1993) showed that this increase in TH activity in LC nerve terminal regions is associated with an increased ability of these neurons to release norepinephrine in response to nicotine, demonstrating a functional consequence of this increase in TH activity. This effect of nicotine on norepinephrine release has also been observed in cultured LC neurons and is at least partially a consequence of agonist occupation of nicotinic acetylcholine receptors on LC cell bodies (Gallardo and Leslie, 1998; Fu et al., 1999). Mitchell et al. (1993) also presented preliminary evidence that nicotine treatment leads to an induction of TH mRNA in the LC. More recently, Serova et al. (1999a) confirmed that when nicotine is administered chronically via either repeated subcutaneous daily injections or chronic subcutaneous infusion, TH mRNA is induced in the LC.

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In a recent report, our laboratory showed that chronic administration of nicotine (two subcutaneous injections per day spaced ~12 h apart) is associated with the induction of TH protein and TH mRNA in rat adrenal medulla (Sun et al., 2003). This response is primarily due to a sustained stimulation of adrenal TH gene transcription rate that occurs only after the animals have been injected with nicotine for at least 3 days. In the present report, we test whether chronic nicotine administration also leads to a sustained stimulation of TH gene transcription rate in the LC. To test this hypothesis, a semiquantitative RT-PCR assay was developed to measure the levels of TH RNA primary transcripts in the LC. A change in the levels of these primary transcripts is an indirect measure of a change in TH gene transcription rate. Using this new assay, we show that chronic nicotine treatment leads to a transient increase in TH gene transcription rate. However, the sustained stimulation of the TH gene observed in the adrenal medulla is not observed in the LC in response to either chronic nicotine treatment or repeated immobilization stress. These results suggest that post-transcriptional mechanisms may also play an important role in maintaining the long-term inductions of TH mRNA and TH protein that occur in the LC after these chronic treatments.

Materials and Methods

Treatment of Rats. Male Sprague-Dawley rats (175–250 g) purchased from Charles River Breeding Laboratories (Portage, MI) were used in this study. Rats were injected subcutaneously with doses of nicotine ranging from 0.4 to 2.3 mg/kg (dosages were expressed in terms of nicotine free base, even though the bitartrate salt was used for injections). A short, mild seizure was observed using 1.6 mg/kg nicotine, whereas longer and more convulsive seizures were noted when using 2.3 mg/kg nicotine. No seizures were observed using 0.8 mg/kg nicotine or lower doses. These injections were made twice daily with injections spaced ~12 h apart. Nicotine bitartrate was dissolved in phosphate-buffered saline (150 mM NaCl and 10 mM potassium phosphate), and the solutions were buffered to pH 7.5. The injections were made using a volume of 1 ml/kg. Control rats were injected with the same volume of phosphate-buffered saline. Rats were administered different numbers of injections ranging from one injection to 29 injections administered over 15 days. The final injection was always administered in the morning. Rats were euthanized using an overdose of sodium pentobarbital (150 mg/kg injected intraperitoneally) at different times after this final injection. Adrenal glands were removed while the animals were anesthetized before death and immediately frozen on dry ice. Brain regions were dissected on ice; these regions included the LC, hippocampus, cerebellum, and frontal cortex.

Immobilizations were performed as described in Nankova et al. (1994). Briefly, the four limbs of each rat were taped to a metal board and the head was restrained in metal rings fixed to the board. The rats were immobilized for 2 h each day in the morning. Immediately after or 24 h after the seventh immobilization, rats were euthanized and tissues were dissected.

All procedures and drug administrations with rats were performed in accordance with the guidelines and approval of the University of Rochester Committee on Animal Resources.

Assays for TH Enzyme Activity and TH Protein. TH activity was assayed as described by Kapatos and Zigmond (1979). Briefly, frozen brain tissues were homogenized in 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 assayed by a coupled decarboxylation assay, under conditions in which enzyme activity was linear with respect to both time and supernatant protein

concentration. The assays were carried out using 4 mM 6-methyltetrahydrobiopterin as cofactor and 0.1 mM [14 C]tyrosine (specific activity 50 Ci/mol) as substrate, which are essentially V_{\max} conditions. Protein was measured by the method of Bradford (Bradford, 1976), using bovine serum albumin as a standard. TH activity was expressed as picomoles of $^{14}\text{CO}_2$ formed per minute per milligram of protein.

TH protein was measured using Western analysis as described in previous publications (Bowyer et al., 1998; Piech-Dumas et al., 1999; Sterling and Tank, 2001). A representative group of the same LC supernatants used for assaying TH activity were used for measuring TH protein. Briefly, for each sample three different concentrations of supernatant protein (10–50 μg) were loaded onto separate lanes of 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. The samples were subjected to electrophoresis, transferred to nitrocellulose, and immunoblotted using rabbit anti-serum specific for TH (Tank et al., 1984). The antibody-TH complexes were detected using the Amersham ECL system (Amersham Biosciences Inc., Piscataway, NJ) 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 micrograms of TH protein per milligram of protein.

Measurement of TH mRNA in the LC Using Semiquantitative RT-PCR. Semiquantitative RT-PCR assays were performed essentially as described in Sun et al. (2003). Briefly, 0.4 μg of LC RNA were 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 [32 P]dATP (0.5 $\mu\text{Ci}/\text{reaction}$) into double-stranded products encoding 519-bp TH cDNA or 295-bp 28S cDNA. The 5' and 3' primers used for the PCR for TH mRNA were selected such that they encoded regions of different exons separated by introns; therefore, PCR products derived from genomic DNA contaminating the isolated RNA would produce a detectably larger PCR band. These bands were never observed.

For each RT reaction, 6 μl of 4 ng/ μl hexamer random primers (Invitrogen, Carlsbad, CA) were added to 2 μl of RNA in a thin-walled PCR tube. The reactions were heated to 70°C for 5 min and then cooled to 5°C to anneal the primers. Subsequently, 12 μl of reaction buffer was added to the reaction mixtures (final volume 20 μl). Final concentrations of reactants were as follows: 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM MgCl_2 , 10 mM dithiothreitol, 0.5 mM dNTPs, 2.5 units/ μl RNase inhibitor and 50 units Superscript II (Invitrogen). The reaction mixtures were warmed to 42°C for 15 min, heated to 99°C for 5 min, and then placed on ice. PCR amplifications of TH and 28S cDNAs were performed in separate reaction tubes. A 2- μl aliquot of the 20- μl RT reaction was used as the template for the PCR amplification. The PCRs were performed in a 50- μl reaction volume containing (final concentrations): 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl_2 , 200 μM dNTPs, 1.5 units of *Taq* DNA polymerase (Invitrogen), 0.2 μM of both 5' and 3' primers, and 0.5 μCi (3000 Ci/mmol) of [α - 32 P]dATP (PerkinElmer Life and Analytical Sciences, Boston, MA). The primers for the PCR amplification of TH and 28S cDNAs were selected using the cDNA sequences for these mRNAs in GenBank (National Cancer Institute/Frederick Biomedical Supercomputing Center, Frederick, MD) and the program OligoR. The 5' TH mRNA sense primer encoded cDNA sequences 390 to 410; the 3' antisense primers were complementary to TH cDNA sequences 888 to 908. The sequences for these primers were as

follows: 5' TH primer, 5'-ccc cac ctg gag tat ttt gtg-3'; 3' TH primer, 5'-atc acg ggc gga cag tag acc-3'. The 5' 28S primer encoded sequences 1 to 22 of 28S cDNA (5'-gtg aac agc agt tga aca tggg-3'); the 3' 28S primer were complementary to 28S cDNA sequences 276 to 295 (5'-aac cgc gac gct ttc caag-3'). All PCRs were performed using an Eppendorf Mastercycler and Invitrogen enzymes and protocols with slight modifications. For TH cDNA amplification, each PCR cycle, with the exception of the first cycle, which had a longer denaturing period at 94°C for 2 min, and the final cycle, which had an extension period of 7 min, consisted of three steps: 1 min at 94°C (denaturing), 1 min at 55°C (annealing), and 1 min at 72°C (primer extension). The number of cycles used for PCR was between 24 and 26, depending upon the assay. After the last PCR cycle the reactions were allowed to cool to room temperature and then subjected to electrophoresis using a 6% nondenaturing polyacrylamide gel, as described in Bowyer et al. (1998). The same protocol was used for PCR amplification of 28S cDNA, except that the number of cycles used was between 14 and 16. For each experiment, the linearity of the RT-PCR for both TH mRNA and 28S rRNA was assessed with respect to both micrograms of RNA added to the RT reaction and the number of PCR cycles. After electrophoresis, the gels were dried down onto Whatman 1MM paper (Schleicher & Schuell, Keene, NH). The levels of ³²P-labeled RT-PCR products separated on the gels were quantified using the Phosphor-Imager system from Amersham Biosciences Inc. after exposure to the phosphor screens for 2 to 24 h. The ImageQuant™ software (Amersham Biosciences Inc.) methods of volume integration were used to quantify the intensity of the RT-PCR bands. Alternatively, the dried-down gels were exposed to X-ray film for 1 to 3 days and the autoradiographic signals were quantified using a scanner and NIH Image software as described above.

Measurement of TH RNA Primary Transcripts Using Semi-quantitative RT-PCR and Primers Directed to Different TH Gene Intron Sequences. These assays were performed essentially as described above, except that PCR primers were chosen to amplify TH genomic intron sequences present in TH RNA primary transcripts. In most experiments, primers directed against sequences within intron-2 of the TH gene were used. The rationale for the choice of primers is presented under *Results* and Fig. 3. The primers used to amplify intron-2 sequences between bases 72 and 234 of intron-2 (with base 1 designated as the first base in intron-2) were as follows: forward primer, 5'-ccagggtctaatcaggctg-3'; and reverse primer, 5'-caggtcactagccagagcaac-3'. In a number of experiments, we also used primers that amplified sequences within either intron-1, intron-5, or intron-12 of the TH gene. The sequences of these primers were designed based on the genomic sequence of the rat TH gene found in GenBank (#NW_047563) and were chosen to produce PCR products of similar size to that amplified by the intron-2 primers. These primers were as follows (the first base of each intron was designated as base 1): intron-1, bases 1 to 169; intron-1, bases 1410 to 1603; intron-5, bases 40 to 201; and intron-12, bases 57 to 232.

For these assays, the RNA samples used for the RT reaction were routinely treated with DNase (1 unit/μg RNA), to remove any contaminating genomic DNA. Furthermore, during the isolation of the total cellular RNA, especial care was taken to avoid contamination of the aqueous RNA solution with DNA from the interface of the organic and aqueous phases during the extraction procedure. Finally, for each sample, two RT reactions were performed, one with and one without RT. PCRs were carried out using the products of both these RT reactions for each sample and the products were run on separate lanes of the gel. If significant amounts of genomic DNA were contaminating the RNA samples, then a PCR product of the predicted size should be observed in the samples that were incubated in the absence of RT. If this occurred, then the samples were repurified and/or retreated with DNase to remove the genomic DNA. An example of this type of analysis is seen in Fig. 6B.

As described above for the RT-PCR assays for TH mRNA and 28S rRNA, preliminary experiments were performed for each experiment to verify that the reactions were performed in the linear range with

respect to both PCR cycle number and RNA input. The intron-containing TH RNA PCR signals were expressed as a ratio of the densities of the TH intron signal divided by the signal for 28S rRNA for each sample.

Nuclear Run-On Assays. PC18 cells were cultured and treated as described in previous studies (Tank et al., 1986; Fossum et al., 1992; Sun and Tank, 2002). The cells were isolated after 1 h of treatment with either 0.1 mM 8-chlorophenylthio-cAMP (8-CPT-cAMP) or 0.1 μM dexamethasone. Nuclear run-on assays were performed as described by Sun and Tank (2002).

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-Newman-Keuls or Dunnett's multiple comparison test. A level of $p < 0.05$ was considered statistically significant.

Results

Chronic Nicotine Treatment Leads to Long-Term Induction of TH Activity and TH Protein in LC Neurons.

Rats were injected twice per day (injections spaced ~12 h apart) for 14 days with saline or different doses of nicotine. The last injection was given on the morning of the 15th day, and the animals were administered an overdose of sodium pentobarbital 3 h after this last injection. The LC, hippocampus, cerebellum, and frontal cortex were dissected from the rat brains, and TH activity was measured in these brain tissues under V_{\max} conditions. There were dose-dependent increases in TH activity in all four brain regions (Fig. 1A). Treatment of the rats with 0.8 mg/kg nicotine was associated with 1.9- and 1.7-fold increases in TH activity in the LC and hippocampus, respectively. Significant increases were not observed in either the cerebellum or frontal cortex using this dose. When the rats were treated with 1.6 mg/kg nicotine, significant and apparently near-maximal increases in TH activity were observed in all four brain regions. Treatment of the rats with 0.4 mg/kg nicotine was not associated with TH induction in any brain region.

In most cases, increases in TH activity measured under V_{\max} conditions are indicative of changes in TH enzyme protein (Tank et al., 1986; Fossum et al., 1991; Piech-Dumas et al., 1999; Sun et al., 2003). However, to verify that these increases in TH activity represented induction of TH enzyme protein, Western analysis was used to measure TH protein directly in a select number of LC supernatant samples that had been used for assaying TH activity. A representative Western blot is shown in Fig. 1B. The antiserum used for this experiment recognizes a single band at ~60 kDa in samples isolated from rodent adrenal medulla, substantia nigra, striatum, or rat pheochromocytoma cells (Tank et al., 1984, 1986; Fossum et al., 1991; Osterhout et al., 1997; Bowyer et al., 1998; Sun et al., 2003). However, when LC supernatant proteins were subjected to Western analysis using this antiserum, three immunopositive bands were observed. The fastest-migrating band comigrated with the purified TH standard (Fig. 1B). When the antiserum was preabsorbed with excess purified TH and then used for the Western blotting, this fastest-migrating band was eliminated, but the two slower-migrating bands were still observed (data not shown). Hence, the fastest-migrating band apparently represented TH protein. The density of this band in different samples was measured and normalized to the amount of LC supernatant protein applied to the gel. Data from these densitometric

analyses are shown in Fig. 1A. TH protein was induced 2.1- or 2.7-fold when the animals were chronically treated with either 0.8 or 2.3 mg/kg nicotine. These increases were almost identical to the increases in TH activity observed in animals treated with these doses of nicotine. We also tried to measure TH protein in the LC terminal regions (hippocampus and cerebellum); however, the amount of TH protein present in these regions was too small to detect without interference from numerous nonspecific protein bands.

We next determined the dependence of this induction of TH protein on the number of nicotine injections. Rats were injected with 1.6 mg/kg nicotine (twice daily with injections spaced ~12 h apart) for different numbers of days. Animals were killed 3 h after the final injection and TH activity was assayed under V_{\max} conditions in the LC, hippocampus, and cerebellum (Fig. 2A). Increases in TH activity were not observed in either the LC or hippocampus until the animals had been injected with nicotine for 15 days (29 injections). TH activity was induced in cerebellum after 7 days (15 injections). Together, these results suggest that nicotine must be injected chronically for at least 7 days to observe changes in TH activity in LC neurons.

The kinetics of deinduction of TH in LC neurons after the nicotine injections were stopped are presented in Fig. 2B. Rats were injected with saline or 1.6 mg/kg nicotine for 14 days and given one final injection on the morning of the 15th day. Animals were killed at different times after this final injection and TH activity was assayed under V_{\max} conditions in LC, hippocampus, and cerebellum. In all three brain regions, TH activity remained significantly elevated for up to 3 days after the final nicotine injection. In the LC nerve ter-

minal regions (hippocampus and cerebellum), TH activity remained elevated for at least 7 days after the final injection. In fact, TH activity remained induced ~3-fold in the hippocampus 10 days after the final nicotine injection.

Chronic Nicotine Treatment Leads to Long-Term Induction of TH mRNA in the LC. In a similar set of studies as those described above, we tested whether the induction of TH protein in LC neurons was associated with an initial induction of TH mRNA. To this purpose, the semiquantitative RT-PCR assay that we had previously developed for measuring TH mRNA in adrenal medulla was modified. The assay was optimized for RNA input and cycle number to measure TH mRNA in total cellular RNA isolated from LC tissue. The levels of 28S rRNA were used for normalization purposes. A representative autoradiogram showing TH and 28S RT-PCR bands obtained from LC samples is shown in Fig. 1C. Standard curves using known amounts of TH RNA and 28S RNA were also generated and the densities of the PCR bands obtained from each sample were compared with those of the standard curves, such that TH mRNA was expressed as the femtomoles of TH mRNA per picomole of 28S rRNA.

Using this assay, a dose-dependent induction of TH mRNA in the LC was observed after chronic nicotine treatment. Rats were injected for 14 days with saline or different doses of nicotine. On the morning of the 15th day, the animals were administered one final injection and then killed 3 h after this last injection. TH mRNA was induced ~1.7-fold in the LC of rats injected chronically with 0.8 mg/kg nicotine; 2.2-fold increases in TH mRNA levels were observed in the LC of rats injected with greater doses of nicotine (Fig. 1A). This dose-

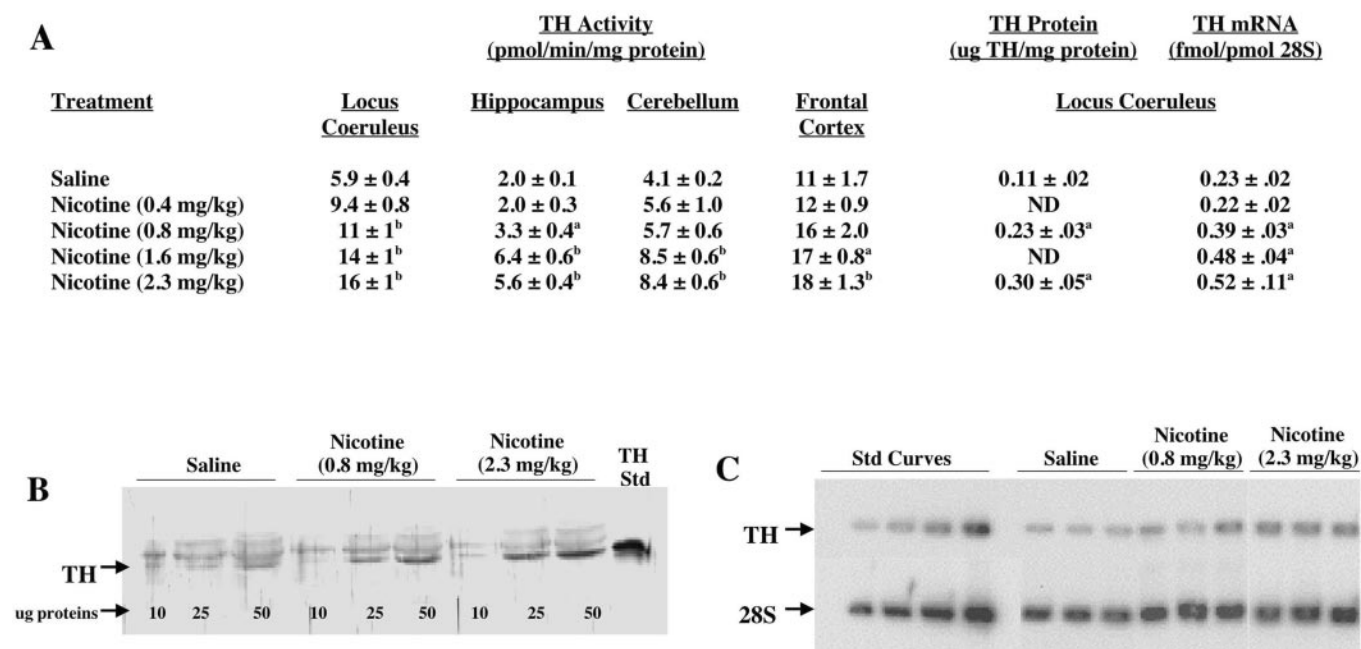


Fig. 1. Effect of long-term repeated nicotine administration on TH activity, TH protein and TH mRNA in LC neurons. **A**, rats were injected (subcutaneously) twice per day with the designated doses of nicotine (administered as nicotine bitartrate salt, but presented as milligrams per kilogram nicotine base). Rats were euthanized with an overdose of sodium pentobarbital 3 h after the last injection of nicotine. TH protein and TH mRNA were assayed in the LC and TH activity (assayed under V_{\max} conditions) was measured in all four brain regions. The results represent the means ± S.E. from five to 10 rats for TH activity and TH mRNA, and from three rats for TH protein. *a*, $p < 0.05$ compared with saline-treated rats. *b*, $p < 0.01$ compared with saline-treated rats. **B**, representative Western blot measuring TH protein in the LC of rats that were chronically administered the designated doses of nicotine. The micrograms of total soluble protein added to each lane of the gel is designated below each lane. The migration of purified TH protein (TH Std) is seen in the right-hand lane. **C**, autoradiogram depicting the products of a representative semiquantitative RT-PCR reaction.

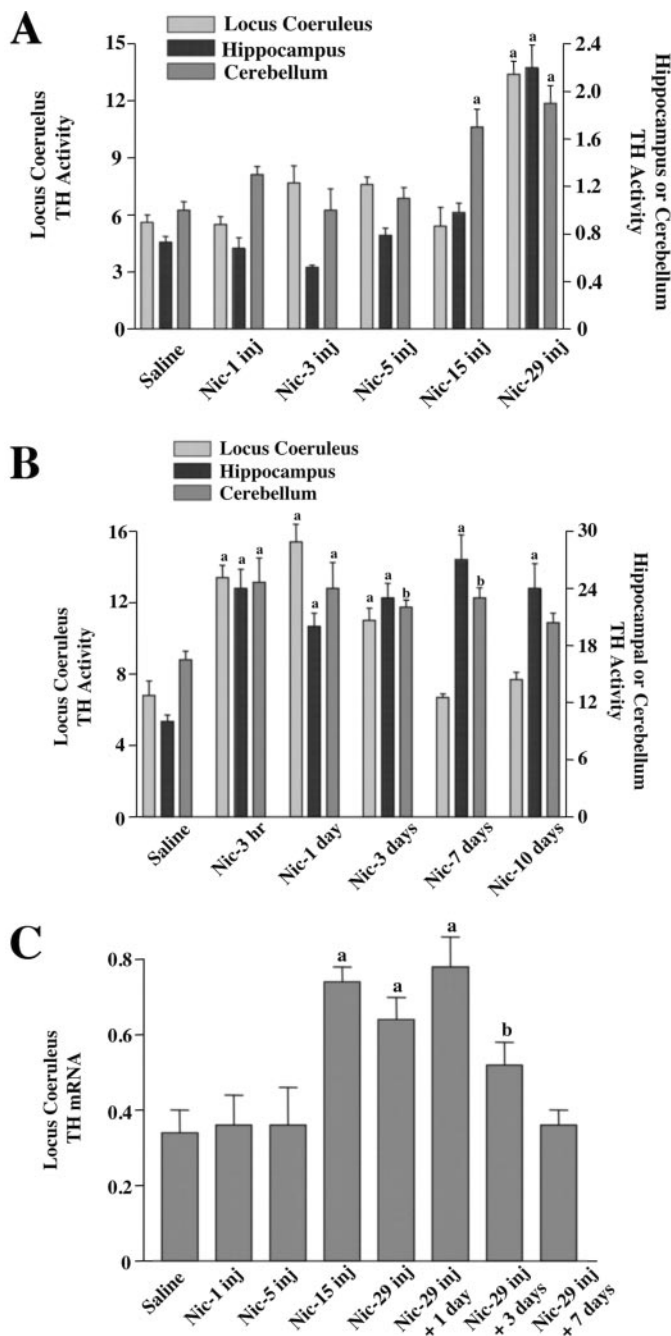


Fig. 2. Long-term induction of TH mRNA and TH protein in response to chronic nicotine treatment. **A**, rats were administered saline or 1.6 mg/kg nicotine twice per day for the number of injections designated in the figure. TH activity was assayed under V_{max} conditions 3 h after the final injection and was expressed as picomoles per minute per milligram of protein. The data represent the means \pm S.E. from five to six rats for the LC and four rats for hippocampus and cerebellum. **B**, rats were administered saline or 1.6 mg/kg nicotine for 14 days (29 injections) and then euthanized at the time points designated in the figure after the last injection. TH activity was assayed under V_{max} conditions and expressed as picomoles per minute per milligram of protein. The data represent the means \pm S.E. from three rats for the LC and six rats for the hippocampus and cerebellum. **C**, rats were administered saline or 1.6 mg/kg nicotine twice per day for the number of injections designated in the figure and unless otherwise noted, euthanized 3 h after the final injection. TH mRNA was measured using the semiquantitative RT-PCR assay and expressed as femtomoles per picomole of 28S. The data represent the means \pm S.E. from five to seven rats. *a*, $p < 0.01$ compared with controls. *b*, $p < 0.05$ compared with controls.

response curve corresponded closely with that obtained for the effect of chronic nicotine on TH activity or TH protein. The dependence of this induction of TH mRNA on the number of nicotine injections is seen in Fig. 2C. TH mRNA levels were not increased when measured 3 h after either one injection or five injections of nicotine. However, after 15 injections (7 days) or 29 injections (14 days) of 1.6 mg/kg nicotine, TH mRNA in the LC was induced ~ 2 -fold. To determine how long TH mRNA levels remained elevated after chronic nicotine treatment, rats were injected 29 times with nicotine and then TH mRNA levels in the LC were measured at different times after the final nicotine injection (Fig. 2C). TH mRNA levels remained induced by ~ 2 -fold at 1 day after the final injection. At 3 days after the final injection, TH mRNA levels were still significantly induced (~ 1.5 -fold). However, by 7 days after the final injection, TH mRNA levels had returned to baseline values.

Development of a Semiquantitative RT-PCR Assay to Measure TH RNA Primary Transcript Levels. The major goal of this study was to determine whether the observed inductions of TH protein and TH mRNA elicited by chronic nicotine treatment were mediated by transcriptional mechanisms. The nuclear run-on assay is the best method to measure changes in gene transcription rates. We successfully used this assay to measure changes in TH gene transcription rate in response to nicotine and other drugs in the rat adrenal medulla (Fossom et al., 1991; Piech-Dumas et al., 1999; Sterling and Tank, 2001; Sun et al., 2003). Serova et al. (1999b) used nuclear run-on assays to demonstrate that immobilization stress is associated with an increase in TH gene transcription rate in rat LC, but they had to pool LC nuclei from eight rats to run a single nuclear run-on assay. Clearly, this assay is too laborious and expensive to perform detailed analysis of the effect of nicotine or other stimuli on TH gene transcription rate in the LC. Consequently, we developed an assay to estimate changes in TH gene transcription rate, using a modification of an approach pioneered by the laboratories of Drs. Sherman and Zigmond (Chang et al., 2000; Sherman and Moody, 1995). These workers used in situ hybridization to measure changes in the levels of TH nuclear RNA primary transcripts that contained sequences present in intron-2 of the TH gene. Assuming that changes in RNA processing do not occur, an increase in the levels of these TH RNA primary transcripts represents an increase in TH gene transcription rate. Based on this approach, a semiquantitative RT-PCR assay was developed to measure changes in the levels of TH RNA primary transcripts in response to nicotine treatment.

In initial experiments, a number of primers directed against different sequences within intron-2 of the TH gene were tested in the PCR reaction (Fig. 3). In these experiments, cDNAs isolated from reverse transcription of either adrenal medulla or LC total cellular RNA were used. It is well established that immobilization stress results in a rapid stimulation of TH gene transcription rate in both adrenal medulla and LC, as assessed using nuclear run-on assays (Nankova et al., 1999; Serova et al., 1999b) or TH gene promoter activity measurements in transgenic mice (Osterhout et al., 1997). Hence, we tested which of these primers was optimal for measuring changes in intron-2-expressing TH RNA primary transcripts elicited by immobilization stress (Fig. 3). Four different primer sets were used. All four

sets produced a single major PCR product of the predicted size using adrenal medulla samples. When using LC samples, multiple PCR products were obtained using all four primer sets. However, only for the 9 to 234 primer set, did these nonspecific PCR products interfere with the measurement of the predicted PCR product that represented the TH RNA primary transcript. Using each of the primer sets, increases in the levels of TH RNA primary transcripts encoding these intron-2 sequences were detected in both adrenal and LC samples derived from immobilized rats. Based on subsequent experiments to optimize cycle number and RNA input, we chose to use the 72 to 234 primers for subsequent assays.

We next tested whether changes in the levels of intron-2-expressing TH RNA precursors correlated closely with changes in TH gene transcription rate measured using nuclear run-on assays. In the first set of studies, rat pheochromocytoma PC18 cells were treated with either 0.1 mM 8-CPT-cAMP or 0.1 μ M dexamethasone; both these compounds stimulate TH gene transcription rate in PC18 cells (Fossom et al., 1992). Cells were treated for 1 h with these inducing agents; nuclei were isolated from one aliquot of cells for nuclear run-on assays, whereas total cellular RNA was isolated from a second aliquot of cells for measurement of intron-2-containing TH RNA precursor levels. Results are presented in Fig. 4A. Both 8-CPT-cAMP and dexamethasone stimulated TH gene transcription rate by 3–5 fold, as determined using either assay. In a second set of experiments, rats were injected once with either 1.6 mg/kg nicotine or 5 mg/kg bethanechol, and adrenal glands were removed under anesthesia 20 min after the injection; both these drugs stimulate TH gene transcription rate in adrenal medulla (Fossom et al., 1991; Piech-Dumas et al., 1999; Sun and Tank, 2003). Total cellular RNA was isolated from each adrenal and RT-PCR was used to measure intron-2-expressing TH RNA precursors. Results are presented in Fig. 4B. Nicotine elicited an \sim 3-fold induction of TH RNA precursor levels, whereas bethanechol produced an \sim 2-fold induction. These increases in TH RNA primary transcript levels correlated closely with the changes in TH gene transcription rate measured using nuclear run-on assays in previous studies (Fossom et al., 1991; Piech-Dumas et al., 1999; Sun et al., 2003).

Finally, we tested whether the increases in TH RNA primary transcript levels were unique to those RNA precursors that expressed intron-2 sequences. For these studies, RNA

isolated from PC18 cells was subjected to RT-PCR using four different sets of PCR primers. These primers were directed toward sequences within intron-1, -2, -5, and -12 of the TH gene. Each set of primers was optimized with respect to cycle number and RNA input and produced a single major band of predicted size (Fig. 5). When normalized to the 28S rRNA signal, the levels of the intron-2-expressing TH RNA primary transcripts were the highest under basal culture conditions. The intron-1-expressing primary transcripts were expressed at a level \sim 3.5-fold lower than the intron-2-expressing primary transcripts; intron-5-expressing and intron-12-expressing primary transcripts were expressed at even lower basal levels. (Note that the autoradiograms in Fig. 5 depicting the PCR products derived from 28S, intron-1 and intron-2 primers were exposed to film for the same period of time, whereas those depicting the results derived from intron-5 or intron-12 primers were exposed for much longer periods of time for illustration purposes). Nevertheless, treatment of the cells with either 8-CPT-cAMP or dexamethasone produced 2–5 fold increases in the levels of TH RNA precursors using each of these primer sets (Fig. 5).

Chronic Nicotine Treatment Leads to Induction of TH RNA Precursor Levels in Rat Adrenal Medulla and LC. This RT-PCR assay was used to detect changes in the levels of TH RNA precursors in the LC and adrenal medulla after chronic nicotine treatment. When rats were administered saline chronically for 14 days and then given a final injection of nicotine on the morning of the 15th day, the levels of intron-2-expressing TH RNA primary transcripts increased rapidly (within 20 min) by \sim 2.5-fold in the adrenal medulla (Fig. 6A). This induction was transient, in that TH RNA precursor levels returned to basal values 24 h after this single nicotine injection. In contrast, when rats were treated with nicotine chronically for 14 days and given a final nicotine injection on the 15th day, adrenal TH RNA precursor levels were increased 1.7-fold 20 min after this final injection and remained significantly elevated for at least 24 h. These rapid increases in TH RNA precursor levels and the sustained increase observed after chronic nicotine treatment agreed closely with our previously published studies that used nuclear run-on assays to measure nicotine-induced changes in TH gene transcription rate in rat adrenal medulla (Fossom et al., 1991; Sun et al., 2003).

The effect of chronic nicotine treatment on TH RNA pre-

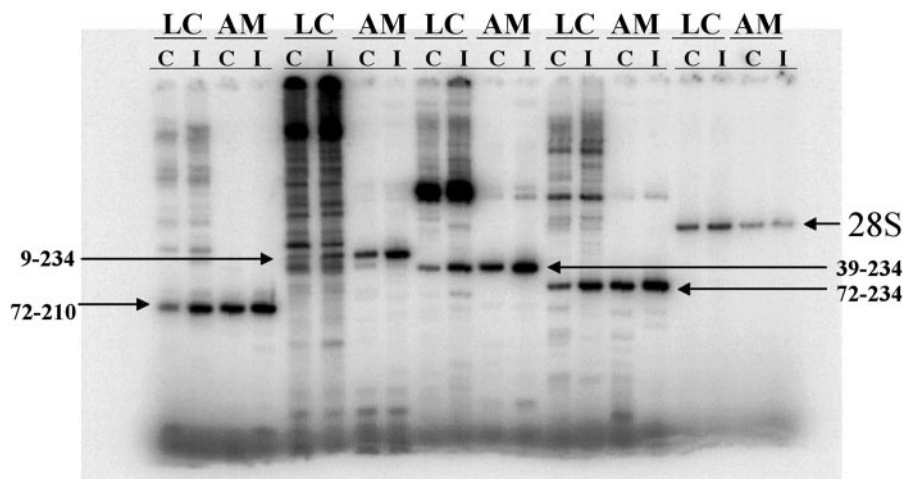


Fig. 3. Development of semiquantitative RT-PCR assay for TH RNA primary transcripts that express intron-2 sequences. Total cellular RNA was isolated from adrenals (AM) and LCs from control rats (C) and rats subjected to a single immobilization (I). TH RNA primary transcripts expressing intron-2 sequences were measured using semiquantitative RT-PCR. The radiolabeled PCR products were separated using polyacrylamide gel electrophoresis and a representative autoradiogram is presented in the figure. The primers used for the PCR reactions designate the intron-2 sequences that were amplified with base 1 denoted as the first nucleotide in intron-2.

cursor levels in the LC is also presented in Fig. 6. An autoradiogram depicting representative results using the intron-2 primers is presented in Fig. 6B. Note that TH intron-2-expressing signals were absent in LC RNA samples that were not reverse transcribed before the PCR, indicating that genomic DNA contamination of the RNA samples was minimal. When the rats were administered saline chronically and given a single nicotine injection on the 15th day, intron-2-containing TH RNA precursors were induced by ~ 1.7 -fold 20 min after the single nicotine injection; the levels of these precursors then returned to basal values by 24 h. This time course agreed with the response observed in the adrenal medulla. However, when the rats were chronically administered nicotine for 14 days and then injected with nicotine on the 15th day, TH RNA precursor levels increased 20 min after the final injection and again returned to basal values by 24 h. This lack of a sustained induction of TH RNA primary transcripts was in contrast to that observed in the adrenal medulla.

To test whether this lack of sustained induction was due to preferential processing of intron-2 sequences at this 24-h time point, we used the primer sets encoding sequences

within intron-1, -5, and -12 to assay TH RNA precursors expressing other intron sequences (Fig. 6C). As observed in the PC18 cells, basal levels of TH RNA primary transcripts expressing these other intron sequences were much lower than those expressing intron-2 sequences. (Note that for the intron-5-expressing TH RNA data, the PCRs were run for two more cycles than those used for amplifying intron-1 or intron-2-expressing TH RNAs). In fact, we were unable to detect significant levels of intron-12-expressing TH RNA precursors in the LC samples. Furthermore, the primers used for measuring intron-1-expressing TH RNA precursors in PC18 cells produced multiple, nonspecific PCR bands that interfered with the measurement of the specific products. Hence, new primers that amplified different sequences with intron-1 (bases 1410–1603) were used for the LC samples in Fig. 6. Using these primers, the nicotine-induced changes in the levels of TH RNA primary transcripts expressing either intron-1 or intron-5 sequences paralleled the changes observed in the intron-2-expressing transcripts. These results reinforce the conclusion that chronic nicotine treatment leads to a rapid, but transient induction of TH RNA primary transcripts in the LC.

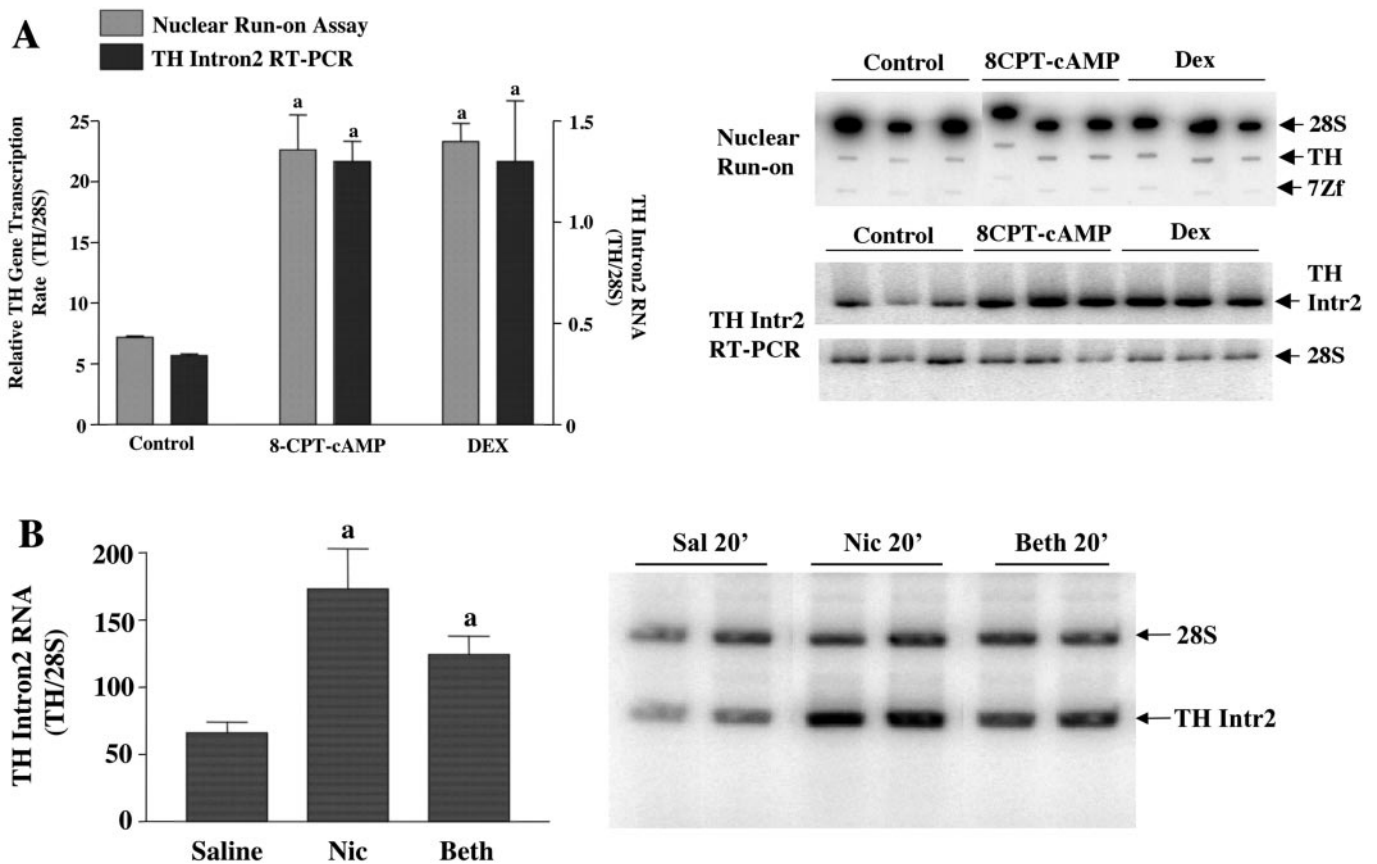


Fig. 4. Measurement of intron-2-expressing TH RNA primary transcript levels in PC18 cells and rat adrenal medulla. **A**, PC18 cells were treated with either 0.1 mM 8-CPT-cAMP or 0.1 μ M dexamethasone for 1 h. Control cells were left untreated. Nuclei were isolated from an aliquot of the cells and nuclear run-on assays were performed. TH gene transcription rate was expressed as the density of the TH signal divided by the density of the 28S signal, after subtraction of the 7Zf signal density from each of these signals. Total cellular RNA was isolated from the remaining cells and semiquantitative RT-PCR was used to measure the levels of intron-2-expressing TH RNA primary transcripts in these RNA samples. TH intron2 RNA level was expressed as the ratio of the density of the TH intron-2 RNA band divided by the density of the 28S rRNA band measured in the same sample. The data represent the means \pm S.E. from three dishes. Autoradiograms depicting the signals derived from the nuclear run-on assay and the RT-PCR assay are presented to the right of the bar graph. **B**, rats were administered subcutaneously either saline, 1.6 mg/kg nicotine, or 5 mg/kg bethanechol, and adrenals were removed under deep anesthesia 20 min after the injection. Total cellular RNA was isolated from the adrenal glands and semiquantitative RT-PCR was used to measure intron-2-expressing TH RNA primary transcript levels. An autoradiogram depicting some of these results is shown to the right of the bar graph. The data represent the means \pm S.E. from three rats. *a*, $p < 0.05$ compared with controls.

Repeated Immobilization Stress Leads to Transient Induction of TH RNA Precursors in Rat LC. The results of these studies suggest that the long-term induction of TH mRNA that occurs in the LC after chronic nicotine treatment is not a consequence of a sustained stimulation of TH gene transcription rate. This result is very surprising, since the long-term induction of TH mRNA in the adrenal medulla is associated with a sustained increase in TH gene transcription rate that persists for up to 7 days after drug administration is ceased (Sun et al., 2003). To test whether this lack of sustained transcriptional activation in the LC is peculiar to the nicotine stimulus or a more generalized response to other types of chronic stimuli, we tested the response of TH gene expression in the adrenal medulla and LC to immobilization stress. It is well established that repeated immobilization stress elicits a sustained induction of TH mRNA in the adrenal medulla, which is associated with a sustained increase in TH gene transcription rate (Nankova et al., 1994, 1999; Osterhout et al., 1997). This adrenal response to repeated immobilization stress is very similar to that observed to chronic nicotine treatment (Sun et al., 2003). Hence, rats were subjected to daily 2-h immobilizations for 7 days and then euthanized either immediately or 24 h after the last immobilization stress. Immobilization stress was associated with a long-term induction of TH mRNA in adrenal medulla (Fig. 7A). This induction was associated with a long-term

induction of intron-2-expressing TH RNA primary transcripts. These results are in excellent agreement with previous reports that used nuclear run-on assays or TH gene promoter assays to show a sustained transcriptional activation of the TH gene after repeated immobilization stress (Osterhout et al., 1997; Serova et al., 1999b).

Repeated immobilization stress also elicited a 3-fold induction of TH mRNA in the rat LC, which was observed immediately after the last immobilization. This result agreed with previous reports by Rusnak et al. (1998, 2001). An ~2-fold induction of LC TH mRNA was still observed 24 and 48 h after the last immobilization episode. Intron-2-expressing TH RNA precursor levels were also induced (4–5-fold) immediately after the last immobilization stress. However, analogous to that observed using chronic nicotine as a stimulus, TH RNA precursor levels returned to basal values by 24 h after the last immobilization.

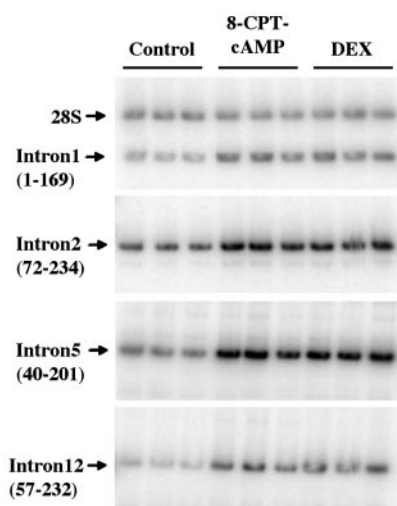
Discussion

Chronic Nicotine Treatment Leads to Long-Lasting Induction of TH Gene Expression in LC Neurons. Dose-dependent increases in TH activity are observed in LC neurons. These increases are dependent on at least 7 days of repeated nicotine injections. Western analyses demonstrate directly (for the first time) that these nicotine-mediated increases in TH activity are due to induction of TH enzyme protein. In LC cell bodies, TH protein levels are elevated for at least 3 days and return to basal values by 7 days after the final drug injection. TH activity remains elevated for longer periods of time in nerve terminal regions, particularly the hippocampus, where enzyme activity is increased 2- to 3-fold for at least 10 days after the final nicotine injection. These results suggest that the effect of chronic nicotine on catecholamine biosynthesis in the regions innervated by LC neurons is very long-lived.

Chronic nicotine administration is also associated with induction of TH mRNA in the LC. TH mRNA is not induced after one, three, or five injections of nicotine; however, after 15 injections (8 days of daily nicotine treatment), TH mRNA is induced by ~2-fold. This induction is maintained for at least 15 days of chronic drug treatment and persists for 3 days after the final nicotine injection. Hence, nicotine administration leads to a sustained induction of TH mRNA in the LC that is dependent on chronic treatment with the drug.

These changes in TH mRNA and TH activity in LC neurons are of the same magnitude as those observed in previous reports (Smith et al., 1991; Mitchell et al., 1993; Serova et al., 1999a). There are some minor discrepancies in the kinetics of these increases between the different studies, which are likely due to differences in dosing regimens. More notably, Mitchell et al. (1993) have reported long-lasting increases in TH mRNA and TH protein, which occur in the LC after a single injection of nicotine. We have not been able to confirm this observation. The results of Serova et al. (1999a) agree with our findings, even though in their hands the induction of TH mRNA requires only five injections of nicotine (two per day spaced 12 h apart). The reasons for these discrepancies with respect to the number of nicotine injections that produce this response remain unclear.

Development of a Semiquantitative RT-PCR Assay to Measure TH RNA Primary Transcripts in the LC. The major question addressed in this report is whether the



Treatment	TH Intron RNA (TH/28S)			
	Intron 1	Intron 2	Intron 5	Intron 12
Control	0.40 ± .03	1.4 ± .1	0.17 ± .01	0.04 ± .01
8-CPT-cAMP	1.4 ± .2 ^a	4.1 ± .3 ^a	0.84 ± .11 ^a	0.21 ± .04 ^a
Dexamethasone	1.2 ± .2 ^a	3.0 ± .5 ^a	0.71 ± .08 ^a	0.18 ± .03 ^a

Fig. 5. Effect of 8-CPT-cAMP or dexamethasone on TH RNA primary transcripts expressing different intron sequences in PC18 cells. PC18 cells were treated with either 0.1 mM 8-CPT-cAMP or 0.1 μ M dexamethasone for 1 h. Control cells were left untreated. Total cellular RNA was isolated from the cells and semiquantitative RT-PCR was used to measure the levels of TH RNA primary transcripts expressing different intron sequences. The primers used to measure these primary transcripts contained intron sequences as shown in the figure with base 1 designated as the first nucleotide within that particular intron. The exposure times for the autoradiograms measuring TH intron-1 and -2, as well as 28S rRNA were ~5 times less than those measuring intron-5 and -12. The data represent the means \pm S.E. from three dishes. a, $p < 0.05$ compared with controls.

sustained induction of TH that occurs in the LC during chronic nicotine administration is mediated by long-lived transcriptional mechanisms. To address this question, a semiquantitative RT-PCR assay was developed to measure changes in the levels of TH RNA primary transcripts in the LC. Implicit in this assay is the premise that a change in the levels of these primary transcripts represents a change in TH gene transcription rate. This RT-PCR assay has many advantages; it is rapid, technically simple to perform, very sensitive and requires isolation of RNA from only a single LC for each measurement. However, there are some limitations that need to be considered.

In our studies, we have routinely used primers to amplify intron-2 sequences to measure changes in TH RNA precursor levels. One problem with this approach is that changes in the

levels of TH RNA precursors containing intron-2 sequences may not be representative of changes in the entire TH RNA precursor pool. To address this potential problem, PCR primers directed against sequences from three to four other TH genomic introns were used to measure stimulus-induced changes in TH RNA precursor levels in PC18 cells or LC. The results of these studies suggest that the increases in the levels of TH RNA primary transcripts that contain intron-2 sequences are representative of the changes that occur in the total pool of these primary transcripts. However, an unexpected finding is that the levels of TH RNA primary transcripts that contain these different intron sequences differ dramatically under basal conditions. These results support the speculation that different introns are spliced from TH primary transcript molecules at different rates, leading to

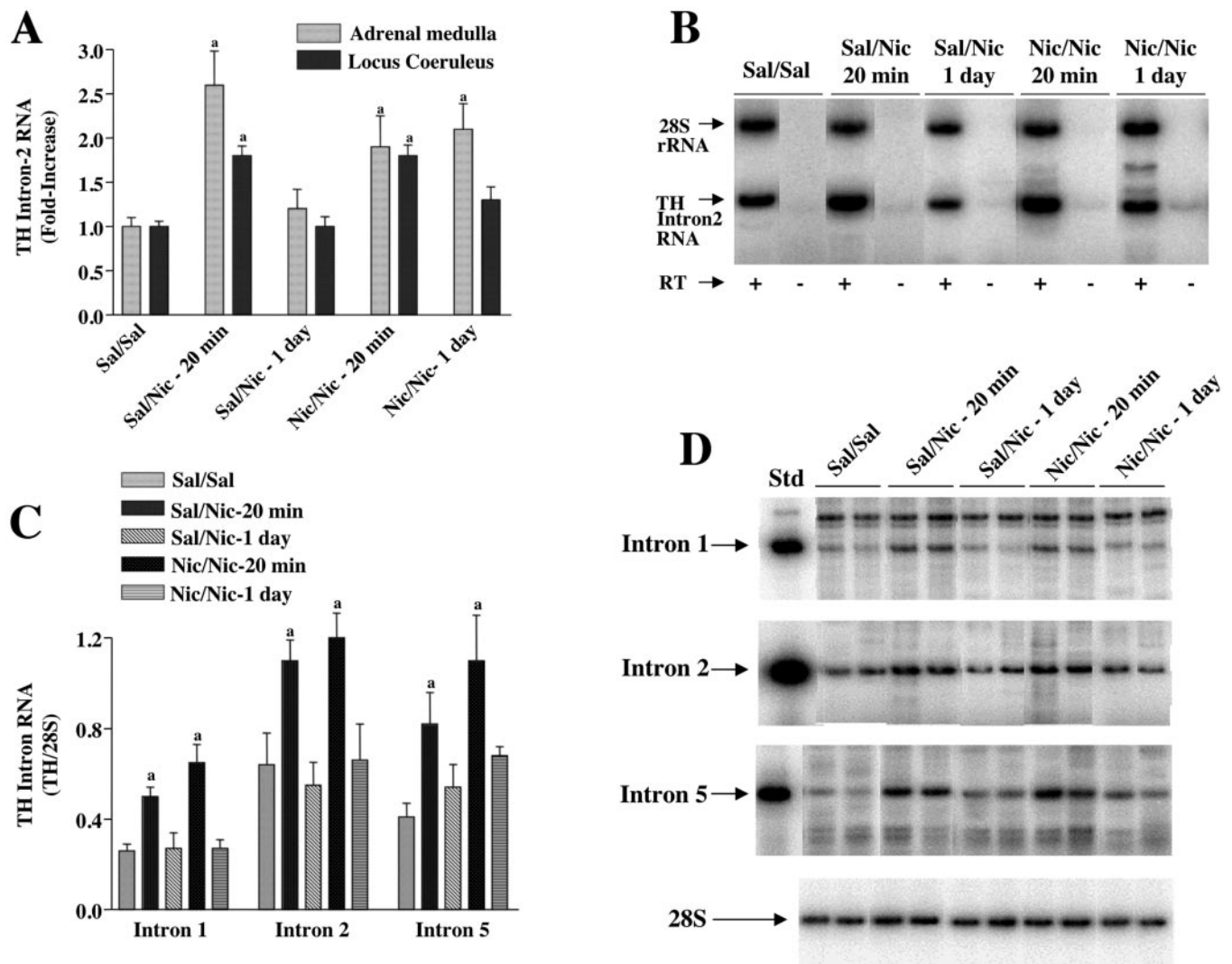


Fig. 6. Nicotine treatment leads to induction of TH RNA primary transcripts in both adrenal medulla and LC. Rats were administered either saline for 14 days (two daily injections spaced ~12 h apart) and given a final injection of either saline (Sal/Sal) or 1.6 mg/kg nicotine (Sal/Nic) in the morning of the 15th day, or 1.6 mg/kg nicotine for 14 days and given a final injection of nicotine (Nic/Nic) in the morning of the 15th day. Adrenal glands and LCs were removed either 20 min or 1 day after this final injection. **A**, bar graph depicting the results of semiquantitative RT-PCR assays (using the 72–234 intron-2 primers) measuring the levels of intron-2-expressing TH RNA primary transcripts in total cellular RNA samples derived from adrenal glands or LCs. The results represent the means \pm S.E. from six rats. **B**, autoradiogram depicting representative results of these RT-PCR assays using RNA samples derived from LC. The assays were performed in the absence or presence of RT in the first step, to verify the lack of significant contamination of the RNA samples with genomic DNA. **C**, bar graph depicting the results of semiquantitative RT-PCR assays using primers that amplify sequences within intron-1, -2, or -5 of total cellular RNA isolated from LC. These primers amplify sequences 1410 to 1603 of intron-1, 72 to 234 of intron-2, and 40 to 201 of intron-5. The data represent the means \pm S.E. from five to six rats for intron-1 and three rats for intron-2 and -5. **D**, autoradiogram depicting representative results of these RT-PCR assays of LC samples. *a*, $p < 0.05$ compared with controls.

different steady-state levels of TH RNA precursors containing these different introns. More work is needed to investigate this possibility.

A second limitation is one of interpretation. This assay is based on the assumption that the changes in TH RNA precursor levels are totally due to changes in their rates of synthesis and that the stimulus does not modulate the rate of processing of these precursors. To our knowledge, there is no evidence that TH RNA precursor processing rate is modified by any stimulus; however, this aspect of TH gene expression has not been investigated. We have addressed this issue by testing whether stimulus-induced changes in TH RNA primary transcript levels correlate with the changes observed in TH gene transcription rate measured using the nuclear run-on assay. Our results indicate a very close agreement using these two assays in both PC18 cells and rat adrenal medulla. These data provide evidence that this assay repre-

sents a reasonably valid estimate of changes in TH gene transcription rate; however, one must be cautious in this interpretation, since stimulus-dependent changes in TH RNA precursor processing may also at least partially mediate the response.

Chronic Nicotine Treatment Transiently Stimulates TH Gene Transcription Rate in the LC. The levels of TH RNA primary transcripts increase rapidly in response to nicotine administration in the LC. These results represent the first demonstration that TH gene transcription rate is enhanced after nicotine administration in the LC. However, this transcriptional response to nicotine is transient, even after chronic nicotine administration (the response is observed at 20 min, but not 24 h after each nicotine injection). This finding is in contrast to that observed in the adrenal medulla (Sun et al., 2003). Adrenal TH gene transcription rate is also rapidly stimulated by nicotine; however, during chronic nicotine treatment, TH gene transcription rate is elevated for a prolonged period of time after the final nicotine injection (at least 3 days after 7 days of chronic nicotine treatment). Our results in the present report support this finding. Our interpretation of these results is that the long-term induction of TH gene expression that occurs in the adrenal medulla during chronic nicotine treatment is a consequence of this sustained transcriptional response. In contrast, the long-term induction of TH that occurs in the LC in response to chronic nicotine is *not* a consequence of sustained transcription.

Since the response of the TH gene to chronic nicotine in these two tissues differs so dramatically, we have tested whether this difference in response is common to another chronic stimulus. The response of the TH gene in the adrenal medulla to either a single or repeated immobilization stress is very similar to that observed after nicotine administration (Sabban and Kvetnansky, 2001). A single immobilization leads to a rapid, but transient stimulation of TH gene transcription rate; repeated immobilization is associated with a sustained transcriptional response (Osterhout et al., 1997; Serova et al., 1999b). Furthermore, File and coworkers (Irvine et al., 1999; Tucci et al., 2002) have shown that at high doses chronic nicotine treatment is anxiogenic and consequently stressful. Hence, we have tested whether a sustained transcriptional response is observed in the LC, as well as the adrenal after repeated immobilization stress. Increases in TH gene transcription rate are observed in both tissues immediately after the seventh immobilization stress. This increase is sustained for at least 2 days in the adrenal, whereas in the LC this transcriptional stimulation is no longer observed at 24 h after the final stress. This lack of a sustained transcriptional response to chronic stimuli in the LC suggests that the mechanisms underlying the long-term induction of TH in LC neurons differ from those mediating the response in the adrenal medulla.

Chronic Nicotine Administration or Repeated Stress May Regulate TH Gene Expression in the LC by Both Transcriptional and Post-Transcriptional Mechanisms. The evidence supporting a transcriptional response to these chronic stimuli in the LC is strong. Our data show a significant increase in TH RNA primary transcript levels 20 min after nicotine administration or immediately after immobilization stress. The simplest interpretation of this result is that TH gene transcription rate increases rapidly, leading

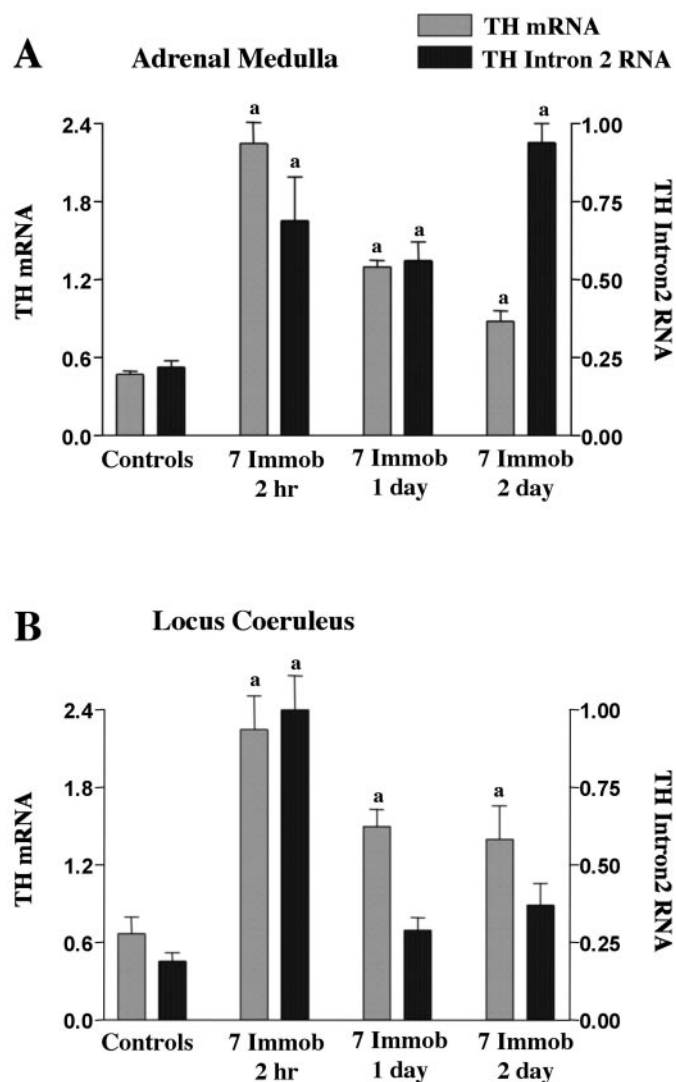


Fig. 7. Repeated immobilization stress induces TH mRNA and TH RNA primary transcripts in adrenal medulla and LC. Rats were subjected to seven daily 2-h immobilizations. Adrenal glands and LCs were removed either 2 h or 1 or 2 days after the last immobilization. Total cellular RNA was isolated from the tissues and semiquantitative RT-PCR was used to measure the levels of TH mRNA and intron-2-expressing TH RNA primary transcripts in these RNA samples. The data represent the means \pm S.E. from four rats. *a*, $p < 0.05$ compared with controls.

to enhanced synthesis of these primary transcripts. This interpretation agrees with previous results (Fossom et al., 1991; Osterhout et al., 1997; Nankova et al., 1999; Sun et al., 2003). However, it is difficult to explain the long-term induction of TH gene expression that occurs in LC neurons in response to chronic stimuli by this relatively short-term transcriptional response. TH mRNA is induced for up to 3 days after chronic nicotine administration in the LC. In contrast, TH RNA primary transcript levels are not elevated at these time points. Similarly, TH mRNA levels in the LC are induced by ~2-fold for at least 48 h after repeated immobilization stress, whereas the levels of TH RNA primary transcripts have returned to baseline values by 24 h. These data are consistent with the hypothesis that post-transcriptional mechanisms play an important role in maintaining the induced levels of TH mRNA in LC neurons after treatment with chronic stimuli. The identification of these post-transcriptional mechanisms requires further investigation.

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