

Long-term Effects of Amphetamine Neurotoxicity on Tyrosine Hydroxylase mRNA and Protein in Aged Rats¹

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ABSTRACT

Four injections (intraperitoneal) of 3 mg/kg amphetamine (2 hr apart) produced pronounced hyperthermia and sustained decreases in dopamine levels and tyrosine hydroxylase (TH) protein levels in the striatum of 15-month-old male rats. A partial recovery of striatal dopamine levels was observed at 4 months after amphetamine. In contrast, TH mRNA and TH protein levels in the midbrain were unaffected at all time points tested up to 4 months after amphetamine treatment. The number of TH-immunopositive cells in the midbrain was also unchanged at 4 months after amphetamine, even though the number of TH-positive axons in the striatum remained dramatically decreased at this time point. Interestingly, TH-immunopositive cell bodies were observed 4 months after amphetamine in the lateral caudate/putamen, defined anteriorly by the genu of the corpus

collosum and posteriorly by the junction of the anterior commissures; these striatal TH-positive cells were not observed in saline- or amphetamine-treated rats that did not become hyperthermic. In addition, low levels (orders of magnitude lower than that present in the midbrain) of TH mRNA were detected using reverse transcription-polymerase chain reaction in the striatum of these amphetamine-treated rats. Our results suggest that even though there is a partial recovery of striatal dopamine levels, which occurs within 4 months after amphetamine treatment, this recovery is not associated with increased TH gene expression in the midbrain. Furthermore, new TH-positive cells are generated in the striatum at this 4-month time point.

Amphetamines are drugs of abuse that affect monoaminergic systems in the brain, particularly dopaminergic nerve terminals in the striatum (Ricaurte *et al.*, 1980; Seiden *et al.*, 1988). The immediate effect of amphetamines is to increase the release of dopamine from these nerve terminals. However, administration of a single high dose or repeated administration or chronic infusion of moderate doses of amphetamines produces neurotoxic effects. These neurotoxic effects have been most extensively studied in the striatum of rodents and are characterized by sustained decreases in striatal dopamine concentration, TH activity and dopamine transport activity (Hotchkiss and Gibb, 1980; Wagner *et al.*, 1980; Bowyer *et al.*, 1992; Eisch *et al.*, 1992). A number of studies have shown that these amphetamine-induced losses in dopaminergic function are at least partially due to the degeneration of striatal nerve terminals and axons, whereas the midbrain cell bodies from which these terminals arise are

relatively spared from damage in rats (Ricaurte *et al.*, 1982; Ryan *et al.*, 1990; O'Callaghan and Miller, 1994). Similar neurotoxic or neuroregulatory effects have been observed in the striatum of primates and humans (Preston *et al.*, 1985; Wilson *et al.*, 1996). The mechanisms responsible for these effects have not been clearly established but may involve interactions among amphetamine-induced hyperthermia, generation of toxic free radicals derived from reactive oxygen species or dopamine itself, depletion of mitochondrial-derived energy supplies and possibly excitatory amino acids (Bowyer and Holson, 1995; Seiden and Sabol, 1995).

The hallmark of amphetamine-induced neurotoxicity is a persistent depletion of striatal dopamine levels. This effect was first reported by Koda and Gibb (1973). They demonstrated that the injection of 15 mg/kg methamphetamine every 6 hr was associated with a ~50% decrease in striatal dopamine levels that lasted for up to 72 hr. Since then, numerous studies using different protocols to administer moderate to high doses of amphetamines have demonstrated this persistent effect in both rats and mice (Ricaurte *et al.*, 1982; Sonsalla *et al.*, 1989; Bowyer *et al.*, 1992; O'Callaghan

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ABBREVIATIONS: TH, tyrosine hydroxylase; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

and Miller, 1994). Furthermore, a number of studies have shown that amphetamines produce a long-lasting decrease in striatal TH activity (Koda and Gibb, 1973; Hotchkiss and Gibb, 1980; Bowyer *et al.*, 1992). These effects have been attributed to the degeneration of dopamine nerve terminals in the striatum. However, in some studies, striatal TH activity and striatal dopamine levels partially recover over several weeks after the initial dopamine depletion produced by the amphetamines (Koda and Gibb, 1973; Bowyer *et al.*, 1992; Ali *et al.*, 1994). This is particularly true when environmental temperature is controlled such that the animals do not become hyperthermic (Bowyer *et al.*, 1992, 1994; Ali *et al.*, 1994). In some instances in which striatal dopamine levels are substantially depleted, this recovery continues for months after exposure to amphetamine or related analogs (Seiden and Sabol, 1995).

Recovery of dopaminergic function has also been reported in the striatum of rats treated with 6-hydroxydopamine (Zigmond *et al.*, 1989a, 1990; Snyder *et al.*, 1990). Dopamine release and biosynthesis are increased in surviving dopaminergic nerve terminals after partial lesions of nigrostriatal neurons using this neurotoxin. Hence, it is reasonable to hypothesize that compensatory changes might also be observed in animals subjected to amphetamine neurotoxicity, particularly because amphetamines damage only nerve terminals, whereas 6-hydroxydopamine destroys both cell bodies and nerve terminals. A number of studies have shown that striatal dopamine levels decrease dramatically 1 to 3 days after methamphetamine administration but that this decrease is partially reversed at 14 days after methamphetamine treatment (Bowyer *et al.*, 1992, 1994; Ali *et al.*, 1994). Similarly, Koda and Gibb (1973) reported that striatal TH activity decreases dramatically 36 hr after methamphetamine administration but returns to normal levels after 72 hr. Some of this recovery may simply be due to newly synthesized TH arriving at the terminals from the cell body, replacing TH that was inactivated or lost during or after methamphetamine exposure. Alternatively, this recovery may be due to up-regulation of TH gene expression in the midbrain nigrostriatal cell bodies after amphetamine administration to compensate for the loss of the striatal nerve terminals. In the present study, we tested this latter hypothesis by measuring TH mRNA and TH protein levels in the midbrain after amphetamine treatment. We also measured striatal TH protein levels to verify that the decrease in TH activity observed in previous studies (Koda and Gibb, 1973; Hotchkiss and Gibb, 1980; Bowyer *et al.*, 1992) is associated with a concomitant change in TH enzyme protein. Finally, we tested whether amphetamine treatment is associated with the production of TH-immunopositive cell bodies in the striatum.

In the present study, we used older rats (15 months old) because amphetamines produce larger neurotoxic effects in the striatum as the animals age and approach senescence. Striatal dopamine levels are depleted by 80% to 90% in aged rats compared with 50% to 70% in 2- to 6-month-old rats (Bowyer and Holson, 1995; Seiden and Sabol, 1995). We reasoned that this increased striatal neurodegeneration in aged animals may accentuate the recovery mechanisms, such as up-regulation of TH gene expression in the midbrain cell bodies.

Methods

Drug administration. Animals used in these experiments were 15-month-old male Sprague-Dawley rats from the NCTR colony. They were individually housed in clear 45 × 22 × 20-cm acrylic cages, with wood chips for bedding with food and water available *ad libitum*. The rats were maintained on a daily 12-hr light/dark cycle, with lights on at 6:00 a.m.; temperature (23 ± 1°C) and humidity (53 ± 15%) were closely controlled. The rats were administered four injections intraperitoneally (once every 2 hr) of 3 mg/kg *d*-amphetamine (calculated as a salt; amphetamine HCl) at an environmental temperature of 24°C. Rats were killed at 1, 3 or 14 days or 4 months after amphetamine treatment. Brains were rapidly removed, and the striatum and midbrain were dissected as described previously (Bowyer *et al.*, 1992). Part of the striatum sample was used to determine tissue dopamine levels, whereas another part was used to isolate total cellular RNA and protein. Midbrain regions were used to isolate RNA and protein for analysis. The substantia nigra was dissected away from most of the ventral tegmental area in a manner such that some of the more later tegmental tissue may have been included. This area of the midbrain was used to isolate total RNA for the RT-PCR and ribonuclease protection assay. Additional rats were killed at 14 days and 4 months for immunohistochemical evaluation.

Isolation and measurement of total cellular RNA. Total cellular RNA was isolated essentially according to the procedure of Chomczynski (1993) and stored at -70°C. Briefly, midbrain and striatum samples were homogenized using 1 ml of Tri Reagent (Molecular Research Center, Cincinnati, OH)/50 mg of tissue. Chloroform was added at 2 parts per 10 parts homogenization buffer, and the mixture was centrifuged for 10 min at 12,000 × *g* to separate the phases and to remove genomic DNA and protein from the RNA in the aqueous phase. The organic phase was saved for determination of TH protein using Western blot analysis (see below). The aqueous phase was brought to 50% isopropanol, and the RNA precipitate was collected by centrifugation. The RNA pellet was washed once with 70% ethanol and once with 100% ethanol, and the final RNA pellet was resuspended in 70 μl of RNase-free H₂O. Ten microliters of the RNA sample from each midbrain was used to measure RNA concentration, 10 μl was used to measure TH mRNA and GAPDH mRNA using the quantitative RT-PCR assay and 50 μl was used to measure TH mRNA and β-actin mRNA using the RNase protection assay.

RNA concentration was estimated by adding a 10-μl aliquot of RNA to 800 μl of RNase-free H₂O and measuring the absorbance at 260 and 280 nm; the ratios of the absorbances at 260/280 nm ranged from 1.6 to 2.0. The following equation was used to calculate RNA concentration:

$$\text{RNA } (\mu\text{g}/\mu\text{l}) = \text{RNA } A_{260} \times 40 \mu\text{g}/\text{ml} \times 0.81 \text{ ml}/10 \mu\text{l}$$

The RNA measurements were determined with the assumption that protein was an additional source of absorbance at A_{260} with an $A_{260/280} = 1$, using the following formula:

$$\text{RNA } A_{260} = ((A_{260}/A_{280}) - 1) \times A_{260}$$

Measurement of TH mRNA and GAPDH mRNA using RT-PCR. To quantify TH mRNA and GAPDH mRNA using RT-PCR, aliquots of 0.4 μg of total cellular RNA were subjected to RT using oligo(dT) primers, random hexamers or 21-nucleotide primers complementary to sequences within the 3' coding region of the specific mRNA. Aliquots of the resulting single-stranded cDNA product were used along with the appropriate primers (see below) in the PCR to incorporate ³²P-dATP into double-stranded products encoding 519-base pair TH cDNA or 626-base pair GAPDH cDNA. This enabled the radiolabeled TH cDNA PCR product to be normalized to the GAPDH cDNA PCR product, as well as to the micrograms of total cellular RNA used in the RT step. The 5' and 3' primers used for the PCR for the two mRNAs were selected such that they encoded regions of different exons separated by at least one intron; therefore, PCR products derived from

genomic DNA contaminating the isolated RNA would produce a detectably larger (>100 base pairs) PCR band. These bands were very rarely observed.

For the RT-PCR, RNase-free water was added to each RNA sample such that the final RNA concentration was 0.2 $\mu\text{g}/\mu\text{l}$. At least two separate RT reactions were performed for each RNA sample. For each RT reaction, 8 μl of 0.6 $\mu\text{g}/\text{ml}$ oligo(dT) 12-18 (Sigma Chemical, St. Louis, MO) was added to 2 μl of 0.2 $\mu\text{g}/\mu\text{l}$ total RNA in a thin-walled PCR tube (GeneAmp[®], Perkin Elmer, Norwalk, CT). The reactions were first heated to 70°C for 5 min and then cooled to 5°C for annealing the oligo(dT) primer to mRNA. When the 3' primer for TH was used instead of oligo(dT), its final concentration in the reaction was 2 μM . Subsequently, 10 μl of reaction buffer was added to the reaction mixtures (final volume = 20 μl), and the reactions were overlaid with 2 drops of mineral oil. Final concentrations of reactants were as follows: 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM concentration of dNTPs, 0.5 units/ μl RNase inhibitor and 50 units of reverse transcriptase. The reaction mixtures were warmed to 42°C for 15 min, heated to 99°C for 5 min and then cooled to 5°C. M-MLV reverse transcriptase or Superscript RNase H⁻ reverse transcriptase (GIBCO BRL, Life Technologies, Gaithersburg, MD) were used for these reactions.

At least two separate PCRs were performed for each RT reaction; this resulted in at least four ³²P-labeled RT-PCR products per mid-brain or striatum. These replicate values were averaged to produce one value ($n = 1$) per rat per brain region as reported in Results. PCR amplifications of TH and GAPDH mRNAs were performed in separate reaction tubes. A 2- μl aliquot of the 20- μl RT reaction solution was used as the first-strand template for the PCR amplification of these mRNAs. The PCRs were performed in a 50- μl reaction volume containing (final concentrations) 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 3 mM MgCl₂, 200 μM concentration of dNTPs, 1.5 units of *Taq* DNA polymerase (GIBCO BRL), 0.2 μM concentration of both 5' and 3' primers and 5 μCi (3000 Ci/mmol) of α -³²P-dATP (DuPont-New England Nuclear, Boston, MA). The primers for the PCR (NBI, Plymouth, MN) amplification of TH and GAPDH mRNAs 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 (5'-390 TH primer) encoded cDNA sequences 390 to 410; the 3' antisense primers were complementary to TH cDNA sequences 888 to 908 (3'-888 TH primer) or 927 to 947 (3'-927 TH primer). The sequences for these primers were 5'-390 TH primer, 5'-ccc cac ctg gag tat ttt gtg-3'; 3'-888 TH primer, 5'-atc acg ggc gga cag tag acc-3'; and 3'-927 TH primer, 5'-ggt gca ttg aaa cac gcg gaa-3'. The 5' GAPDH sense primer encoded cDNA sequences 298 to 318; the 3' antisense primer was complementary to GAPDH cDNA sequences 923 to 943 (GAPDH sense primer, 5'-gct gag tat gtc gtg gag tct-3'; GAPDH antisense primer, 5'-cca gcc cca gca tca aag gtg-3').

All PCRs were performed in thin-walled PCR tubes from GeneAmp[®] using a Perkin/Elmer model 480 DNA thermal cycler. All PCRs used GIBCO BRL enzymes and protocols with slight modifications. For TH mRNA 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 varied from 15 to 35 (see Results). After the last PCR cycle, the reactions were cooled to 5°C. The same protocol was used for PCR amplification of GAPDH mRNA, except that the annealing temperature was 57°C. When PCR was used to detect striatal TH mRNA, two coupled PCRs of 21 cycles each were used. The first PCR used the sense 5'-390 and antisense 3'-927 TH primers. Five microliters of this first PCR product was used in the second PCR, which contained ³²P-dATP and the same sense TH primer used in the first PCR (5'-390) but a different antisense primer, 3'-888 TH primer.

After PCR, the mineral oil was removed, and 10 μl of 6 \times loading buffer (0.25% xylene cyanol FF, 0.25% bromophenol blue and 15% Ficoll) was added to each 50- μl PCR to prepare it for electrophoresis on a 6% nondenaturing polyacrylamide gel. Between 6 and 12 μl of the RT-PCR products were loaded per lane for isolation of radioactive bands. Each gel was run for 3 hr at 25 mA, such that the PCR products traveled at least 6 cm from the origin, and then the gel was dried down onto 15 \times 15 cm Gel Blot Paper (Schleicher & Schuell, Keene, NH). The levels of ³²P-labeled RT-PCR products separated on the gels were quantified using the PhosphorImager system from Molecular Dynamics (Sunnyvale, CA) after exposure to the phosphor screens for 2 to 6 hr. The ImageQuant software (Molecular Dynamics) methods of volume integration were used to quantify the intensity of the RT-PCR bands. In addition, the RT-PCR products in many of the gels were isolated and counted using liquid scintillation spectrometry; this enabled the calculation of pmol of RT-PCR product formed from the PhosphorImager analyses. In some instances, 2% agarose gels were used to separate RT-PCR products for ethidium bromide visualization.

Measurement of TH mRNA using RNase protection assays.

Total cellular RNA isolated from either midbrain (5–10 μg) or striatum (20–30 μg) was used in the RNase protection assay. Antisense riboprobes complementary to TH mRNA or β -actin mRNA were used to detect their respective mRNAs in solution hybridizations. β -Actin mRNA signals were used to control for differences in RNA input into the hybridization reactions and for recovery of RNA duplexes during the procedure. Antisense riboprobes were synthesized using [³²P]UTP according to the manufacturer (Promega, Madison, WI). A nonradioactive sense riboprobe encoding TH sequences complementary to the antisense TH riboprobe was also synthesized using these standard procedures. All riboprobes were purified on a denaturing 5% polyacrylamide/8 M urea gel before use. For TH mRNA antisense and sense riboprobes, pTH.3 was used as a template; this plasmid contained a 280-base pair insert encoding the 3' region of rat TH mRNA (nucleotides 1241–1520) inserted into the multiple cloning site of pGEM3 (Fossom *et al.*, 1991). The β -actin mRNA antisense riboprobe was synthesized using a template containing 125 base pairs of rat β -actin cDNA; this cDNA template was purchased from Ambion (Austin, TX). Hybridizations were performed for 16 to 20 hr at 42° to 45°C in the presence of 5 to 10 μg of total cellular RNA, 500 pg of rat TH antisense riboprobe, 500 pg of rat β -actin antisense riboprobe, 80% deionized formamide, 300 mM sodium acetate (pH 6.4), 100 mM sodium citrate and 1 mM EDTA. Other reaction tubes contained known amounts (0–20 pg) of sense TH riboprobe and 10 μg of total cellular RNA isolated from rat liver in place of the rat brain RNA samples; these reactions were used to construct a standard curve. After hybridization, unhybridized RNA was digested with a mixture of RNase A (5 units/ml) and RNase T1 (200 units/ml) for 30 min at 37°C. The protected radiolabeled RNA duplexes were precipitated by bringing the reactions to a final concentration of 2 M guanidine HCl, 8 mM sodium citrate (pH 7.0), 0.2% L-lauroylsarcosine, 32 mM β -mercaptoethanol and 50% isopropanol. The precipitates were collected by centrifugation at 12,000 $\times g$ for 15 min. The pellets were suspended in 5 μl of a loading buffer containing 80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue and 2 mM EDTA and separated on a 5% nondenaturing polyacrylamide gel. The radiolabeled RNA duplexes were detected autoradiographically or with the use of the PhosphorImager as described above. Radioactive bands representing protected RNA species were densitometrically quantified by scanning the autoradiogram with a Hewlett-Packard ScanJet 4C scanner with a transparency adaptor along with computer-assisted imaging analysis using NIH Image software or by using the ImageQuant software for analyzing phosphorimages as described above. Care was taken to use only those density values within the linear range of the PhosphorImager or the autoradiographic film. Both these quantitative densitometric analyses yielded similar results. The density units obtained for the TH mRNA duplex bands were normalized to the density units obtained for the standard

curve using known amounts of TH sense riboprobe to calculate the picograms of TH mRNA present in the hybridization reactions. These values were converted to attomol (10^{-18} Mol) of TH mRNA and then normalized to femtomoles of β -actin mRNA, which were calculated from the density units obtained for the β -actin mRNA duplex bands in the same samples.

Measurement of TH protein by Western blot analysis. The protein samples were isolated from the organic phase of the RNA extractions described above according to the method of Chomczynski (1993). Protein concentrations were measured using the method of Lowry *et al.* (1951). For each sample, three different concentrations of protein (5, 10 and 25 μ g for striatum and 10, 25 and 50 μ g for midbrain) 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 then subjected to electrophoresis, transferred to nitrocellulose and immunoblotted using rabbit antiserum specific for TH essentially as described by Fossom *et al.* (1991). The only modification of this previously published procedure was that the Amersham ECL system was used to detect the antibody-TH complexes using autoradiography. The autoradiographic bands were quantified by scanning the autoradiograms as described in the previous section and using NIH Image software to calculate the density units. As mentioned above, 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 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 μ g of TH protein/mg of protein loaded onto the gel.

Measurement of striatal dopamine levels. Dopamine and its metabolites were measured using high-performance liquid chromatography with a reverse-phase Supelcosile LC-18 column and electrochemical detection (Bowyer *et al.*, 1994). A mobile phase (pH 3.0) consisting of 70 mM KH_2PO_4 , 1 mM sodium 1-heptanesulfonate, 0.1 mM EDTA and 8% methanol was used to separate dopamine from dihydroxyphenylacetic acid, homovanillic acid, 5-hydroxytryptamine and 5-hydroxyindolacetic acid.

Immunohistochemical localization of TH. At 14 or 120 days after amphetamine treatment, some rats were perfused for histological processing. Rats were administered 60 mg/kg pentobarbital *i.p.*, and after they had reached deep anesthesia, they were perfused transcardially with 35 ml of normal saline and then with 300 ml of 10% formalin in 0.1 M phosphate buffer. Brains were further fixed for 1 to 2 days using 20 ml of the fixative used in the perfusion and then serially sectioned at a thickness of 30 μ m in a coronal plane. TH immunoreactivity in dopaminergic terminals and axons in the striatum and cell bodies in the midbrain was localized using primary antibody to TH (1:2000 dilution) using methods similar to those described by Hsu *et al.* (1981). The avidin and biotinylated horseradish peroxidase macromolecule complex (ABC, Vector Laboratories, Burlingame, CA) procedure was used for visualization of terminals and cell bodies. The TH primary antibody was the same as that used for the Western analysis.

In some experiments, the number of TH-positive cells were counted in sections that included the midbrain isolated from saline- and amphetamine-treated rats. Coronal sections at ~ -5.2 to -5.3 mm relative to bregma [based on the atlas of Paxinos and Watson (1986)] were analyzed. Cells in both the substantia nigra pars compacta and ventral tegmental area were included in the analysis. Sections were visualized at 10 \times under a microscope, and two methods were used to count the number of TH-positive cells in each section. For the first method, the image was enlarged onto a screen. The sections and TH-positive cells were then drawn onto a piece of paper, and the number of cells were manually counted for each section. For the second method, the images were captured on a Macintosh Power PC using NIH Image software, and the images composing each section were made into a composite encompassing

the entire section using Adobe Photoshop. Individual TH-positive cells were marked with a circle of a known density. Using NIH Image software, the density of the circles was used to determine the number of TH-positive cells. Both methods yielded essentially identical results. At least two adjacent sections from each animal were analyzed, and the values obtained from these two sections were averaged and included in the analysis as $N = 1$.

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-Neuman-Kuels multiple comparisons test. A level of $P < .05$ (two-tailed) was considered statistically significant.

Results

Effects of Amphetamine on Striatal Dopamine Levels and Striatal TH Protein

Rats were administered four injections of 3 mg/kg *d*-amphetamine *i.p.* (calculated as the salt, amphetamine:HCl) once every 2 hr at an environmental temperature of 24°C. Core body temperatures were monitored at least every hr. More than 80% of these rats developed severe hyperthermia (body temperatures of $>41^\circ\text{C}$). When their body temperature reached $\geq 41.5^\circ\text{C}$, rats were cooled with crushed ice for 10 to 25 min to prevent lethality. All of the biochemical analyses in the present study were performed only on animals that experienced body temperatures of $>41^\circ\text{C}$, unless otherwise stated. This amphetamine treatment produced marked depletions in striatal dopamine content (table 1). Striatal dopamine levels were decreased by 75% at 1 day after amphetamine and by 80% to 85% at 3 and 14 days after amphetamine. By 4 months after amphetamine, striatal dopamine levels partially recovered; however, they were still reduced by $\sim 55\%$.

Western analysis was used to test whether the decreases in striatal dopamine levels were associated with decreases in striatal TH protein levels. As shown in figure 1, one major protein band was recognized in striatal protein extracts by the TH antiserum; this major immunoreactive protein comigrated with a purified TH standard (lane 7 in fig. 1). Visual inspection of the autoradiograms indicated that striatal TH protein was dramatically reduced in amphetamine-treated rats (see fig. 1 for a representative autoradiogram depicting

TABLE 1

Effect of amphetamine treatment on striatal dopamine and TH protein levels

Rats were administered saline or 3 mg/kg amphetamine four times once every 2 hr and killed at the designated times. Striatal dopamine levels were determined by HPLC, and striatal TH protein levels were determined by Western blot analysis. Data represent the mean \pm S.E. from 6 to 8 rats.

| Treatment | Time after amphetamine | Striatal dopamine | Striatal TH protein |
|-------------|------------------------|---------------------------|--------------------------------------|
| | days | pmol/mg of tissue | $\mu\text{g/mg of cellular protein}$ |
| Saline | 1 | 91 \pm 4 | N.D. |
| Amphetamine | 1 | 24 \pm 4 ^a | N.D. |
| Saline | 3 | 93 \pm 3 | N.D. |
| Amphetamine | 3 | 15 \pm 3 ^a | N.D. |
| Saline | 14 | 84 \pm 4 | 2.1 \pm 0.2 |
| Amphetamine | 14 | 18 \pm 3 ^a | 0.8 \pm 0.1 ^a |
| | months | | |
| Saline | 4 | 106 \pm 4 | 1.7 \pm 0.1 |
| Amphetamine | 4 | 47 \pm 3 ^{a,b} | 0.8 \pm 0.1 ^a |

^a $P < .001$ compared with time-matched saline-treated rats.

^b $P < .01$ compared with amphetamine-treated rats at 1, 3 or 14 days.

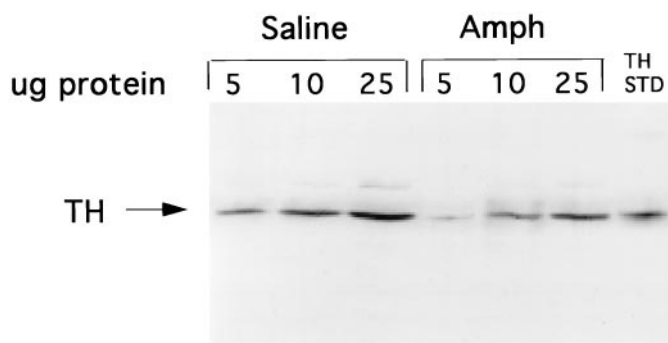


Fig. 1. Western analysis of TH protein in rat striatum. TH protein was measured in the striatum of saline-treated (lanes 1–3) and amphetamine-treated (lanes 4–6) rats. The autoradiogram depicts striatal samples isolated from animals 14 days after amphetamine. For each sample, three different concentrations (5, 10 and 25 µg as designated in the figure) of striatal protein were loaded onto separate lanes of the gel. A known amount of purified TH protein standard was loaded onto lane 7 and was used to normalize signals between gels.

a 14-day time point). The densities of the striatal TH protein bands obtained from 6 to 8 animals were quantified by using either a PhosphorImager or scanning densitometry as described in Methods. These data are tabulated in table 1. Striatal TH protein levels were lowered by ~62% at 14 days after amphetamine and by ~53% at 4 months after amphetamine.

Effect of Amphetamine Treatment on TH mRNA Levels in the Substantia Nigra

Because amphetamine produced a long-lasting decrease in striatal TH protein, we tested whether TH mRNA and TH protein were induced in the midbrain cell bodies to compensate for this reduction in the striatal enzyme. We used two methods to measure TH mRNA: quantitative RT-PCR and RNase protection assays.

RT-PCR assay of midbrain TH mRNA. Using the TH primers, 5'-390 and 3'-888, RT-PCR produced a 519-bp cDNA product encoding nt 390 to 908 of TH mRNA. In extensive preliminary experiments, we verified that the amount of the 519-bp PCR product was independent of the primer [oligo(dT), random hexamers or 3'-TH primer] used in the RT step; hence, in most experiments, we used oligo(dT) primers. The production of this 519-bp PCR product was exponential for up to 25 PCR cycles, when 0.4 µg of total cellular RNA was used for RT (fig. 2, top). Exponential increases were observed when either oligo(dT) or the 3'-888 TH primer was used in the RT step. Furthermore, using 21 PCR cycles, the production of this TH PCR product was linear, when using between 0.05 and 0.8 µg of total cellular RNA from midbrain in the RT reaction (fig. 2, bottom). For quantitative determination of midbrain TH mRNA levels, 21 PCR cycles were sufficient to generate a strong phosphorimage after a 3-hr exposure, when either oligo(dT) or the 3'-888 TH primer was used for the RT (fig. 3 A and B). Steps to ensure the exponential (PCR) and linear (RT) amplification of products [generated with oligo(dT) primers] for GAPDH mRNA were identical to those that were used for TH mRNA. Using the same RT reaction products that were used for PCR quantification of TH mRNA, exponential increases in the 626-bp GAPDH RT-PCR products were attained for up to 23 PCR cycles (fig. 2, top). When 19 PCR cycles were used to generate the

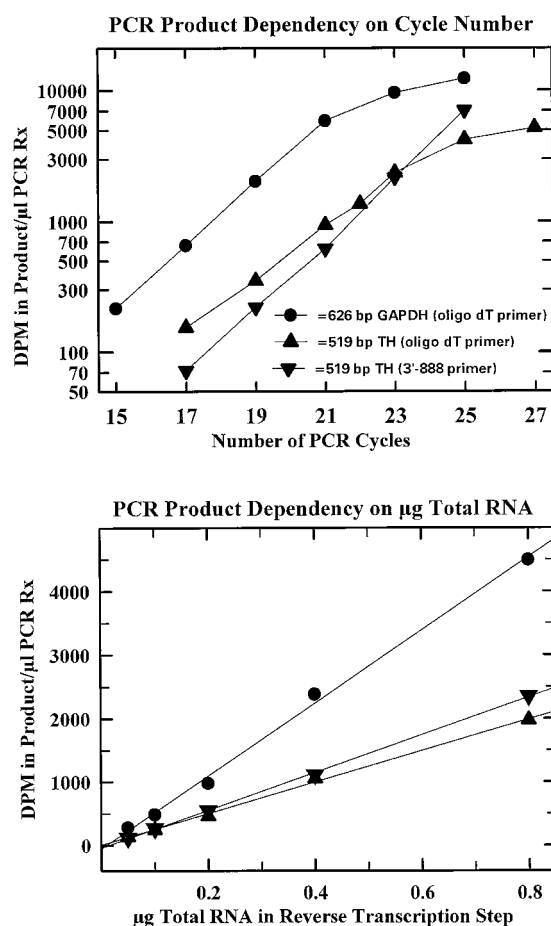


Fig. 2. Measurement of midbrain TH mRNA levels using quantitative RT-PCR. This figure demonstrates that the syntheses of the 519-bp TH cDNA and 626-bp GAPDH cDNA RT-PCR products were dependent on the number of cycles used in the PCR reaction (top) and the amount of total cellular RNA used in the RT reaction (bottom).

GAPDH RT-PCR product, linearity was observed for 0.05 to 0.8 µg of total RNA input into the RT reaction (fig. 2, bottom). The GAPDH RT-PCR products (fig. 3C) were used to normalize the TH RT-PCR products.

The production of radiolabeled TH and GAPDH RT-PCR products, as well as the ratio of TH to GAPDH RT-PCR products, are presented in table 2. These results indicate that the levels of midbrain TH mRNA and GAPDH mRNA were relatively constant in controls over the 1-day to 4-month time course. It should be noted that the actual ratio of TH mRNA to GAPDH mRNA was calculated to be between 0.1 to 0.2 because only 19 PCR cycles were used for GAPDH compared with 21 cycles for TH. Only minor, and insignificant, differences in TH mRNA levels were detected at all time points in amphetamine-treated animals. This result was also apparent when comparing the control versus amphetamine-treated phosphorimages at various time points in figure 3. Midbrain GAPDH mRNA levels also did not significantly differ between control *vs.* amphetamine-treated rats. These results suggest that TH mRNA levels were not modulated by amphetamine treatment.

RNase protection assay of midbrain TH mRNA. To confirm this conclusion, we measured TH mRNA with the more established and quantitative technique of solution hybridization followed by RNase digestion, using the same mid-

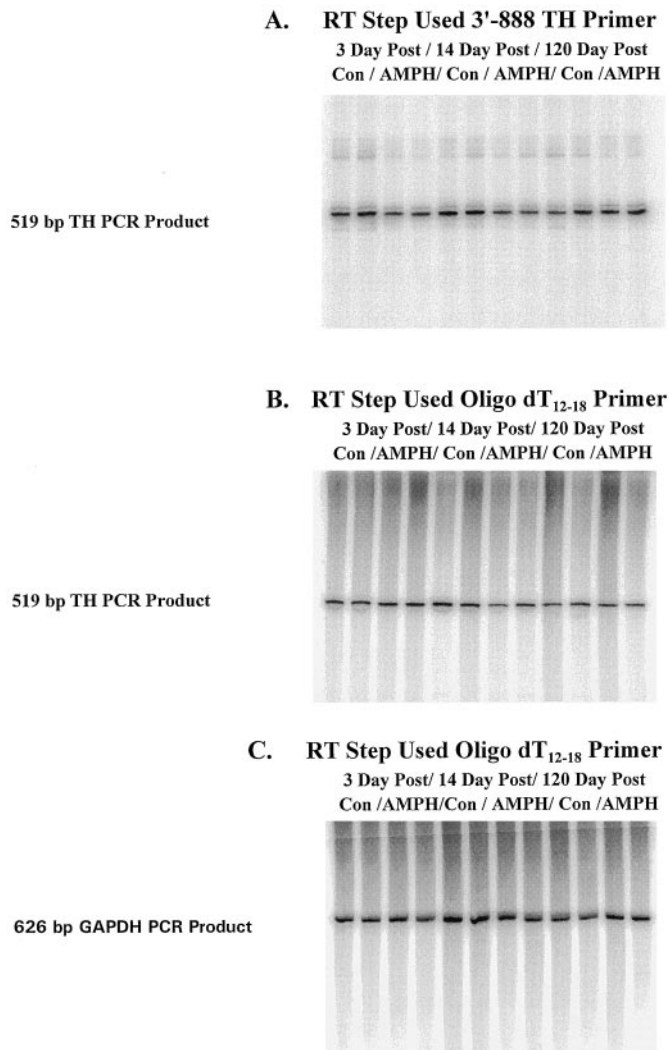


Fig. 3. Measurement of TH mRNA in midbrain samples from saline-treated and amphetamine-treated rats using RT-PCR. Rats were treated with saline or amphetamine, and total cellular RNA was isolated from the midbrain. PCR products were isolated on 5% nondenaturing acrylamide gels, and the radioactive bands were quantified using phosphorimaging. A, Radiolabeled RT-PCR products (519 bp) encoding TH cDNA that were derived from midbrain RNA using the specific 3'-888 TH primer in the RT step and 21 cycles for the PCR from control or amphetamine-treated rats at 3 days (lanes 1-4), 14 days (lanes 5-8) or 4 months (lanes 9-12) after amphetamine. B, Radiolabeled RT-PCR products (519 bp) encoding TH cDNA that were derived from the same midbrain RNA sample used in A using oligo(dT) as a primer for the RT step and 19 cycles for the PCR from control or amphetamine-treated rats at 3 days (lanes 1-4), 14 days (lanes 5-8) or 4 months (lanes 9-12) after amphetamine. C, Radiolabeled RT-PCR products (626 bp) encoding GAPDH cDNA that were derived from the same RT used in A and B using the oligo(dT) primer and 21 PCR cycles from control or amphetamine-treated rats at 3 days (lanes 1-4) 14 days (lanes 5-8) or 4 months (lanes 9-12) after amphetamine.

brain RNA samples used for the RT-PCR assay (fig. 4 and table 3). These RNase protection assays yielded results similar to the RT-PCR assays. When normalized to the micrograms of cellular RNA used in the hybridizations, neither midbrain TH mRNA nor midbrain actin mRNA levels in amphetamine-treated rats differed significantly from those in time-matched control rats (table 3). Furthermore, no differences were observed when TH mRNA levels were normalized to actin mRNA levels (table 3, last column). This lack of effect of amphetamine on midbrain TH mRNA and actin

mRNA levels was also apparent when comparing the autoradiographic images (fig. 4).

Effect of Amphetamine on TH Protein Levels in the Midbrain

Even though midbrain TH mRNA levels did not apparently change, it was possible that midbrain TH protein levels might be modulated by amphetamine treatment. Hence, we used Western analysis to measure midbrain TH protein levels. As seen in table 4, midbrain TH protein levels in amphetamine-treated rats did not significantly differ from those measured in control rats at all time points. This lack of effect of amphetamine on TH protein levels was also apparent by visual inspection of the autoradiograms. At the same time point (14 days after amphetamine) at which TH protein levels were reduced in the striatum (fig. 1), TH protein levels were unaffected in the midbrain (fig. 5).

Effect of Amphetamine on the Number of TH-Positive Cells in Rat Midbrain

The lack of effect of amphetamine on TH mRNA and TH protein levels in the midbrain can be interpreted in two ways. One interpretation is that amphetamine did not modulate TH gene expression in midbrain dopaminergic cell bodies, even though striatal TH protein was reduced after amphetamine treatment. The second interpretation is that the number of dopaminergic cell bodies in the midbrain was reduced due to amphetamine's neurotoxic effects, and consequently, the levels of TH mRNA and TH protein per dopaminergic cell body were increased. Most previous studies have demonstrated that neurotoxic doses of amphetamines cause degeneration of dopaminergic nerve terminals and axons in the striatum and nucleus accumbens, without affecting the cell bodies in the substantia nigra and ventral tegmental area (Ricaurte *et al.*, 1982; Ryan *et al.*, 1990; O'Callaghan and Miller, 1994). However, Sonsalla *et al.* (1996) recently presented evidence that methamphetamine produces neurotoxic effects on dopaminergic cell bodies in the mouse midbrain. Hence, we tested whether the amphetamine treatment used in this study produced a decrease in the number of TH-positive cell bodies in the midbrain.

Initial visual inspection of coronal sections cut through the entire midbrain region and immunostained for TH protein indicated that there was no dramatic reduction in TH-positive cell bodies in rats treated with amphetamine at any time point. To test whether amphetamine produced a small change in the number of dopaminergic cell bodies, which was not evident by simple visual inspection, we counted the number of TH-positive cells in representative sections (at -5.3 to -5.2 mm from bregma) of midbrain in saline- *vs.* amphetamine-treated rats at the 4-month time point. Cell bodies in both the substantia nigra pars compacta and ventral tegmental area were summed together in this analysis. Cell counts were performed by either manually counting the number of cells in each section or by computer-assisted analysis of the number of cells in each section. Both procedures yielded similar results. There were no significant differences between the number of TH-positive cells in these sections in the saline- *vs.* amphetamine-treated animals (saline treated, 481 ± 26 cells/section; amphetamine treated, 444 ± 20 cells/section; $n = 7$ animals).

TABLE 2

Effects of amphetamine on TH and GAPDH mRNA in midbrain as determined by RT-PCR

Rats were treated with saline or amphetamine, and midbrain RNA was isolated at the time points. TH mRNA and GAPDH mRNA were measured using RT-PCR. The TH PCR product was produced using 21 cycles, whereas the GAPDH PCR product was produced using 19 cycles.

| Treatment | Time after amphetamine | TH PCR product | GAPDH PCR product | TH/GAPDH |
|-------------|------------------------|---------------------------------------|-------------------|-----------------|
| | days | pmol / 10 μ l of reaction mixture | | |
| Saline | 1 (5) | 98 \pm 17 | 139 \pm 16 | 0.70 \pm 0.1 |
| Amphetamine | 1 (5) | 75 \pm 17 | 97 \pm 16 | 0.78 \pm 0.1 |
| Saline | 3 (7) | 98 \pm 15 | 149 \pm 13 | 0.65 \pm 0.09 |
| Amphetamine | 3 (8) | 85 \pm 14 | 110 \pm 13 | 0.87 \pm 0.08 |
| Saline | 14 (7) | 96 \pm 15 | 164 \pm 13 | 0.55 \pm 0.09 |
| Amphetamine | 14 (8) | 71 \pm 14 | 140 \pm 13 | 0.50 \pm 0.08 |
| | months | | | |
| Saline | 4 (7) | 91 \pm 15 | 120 \pm 13 | 0.78 \pm 0.09 |
| Amphetamine | 4 (8) | 90 \pm 14 | 131 \pm 13 | 0.67 \pm 0.08 |

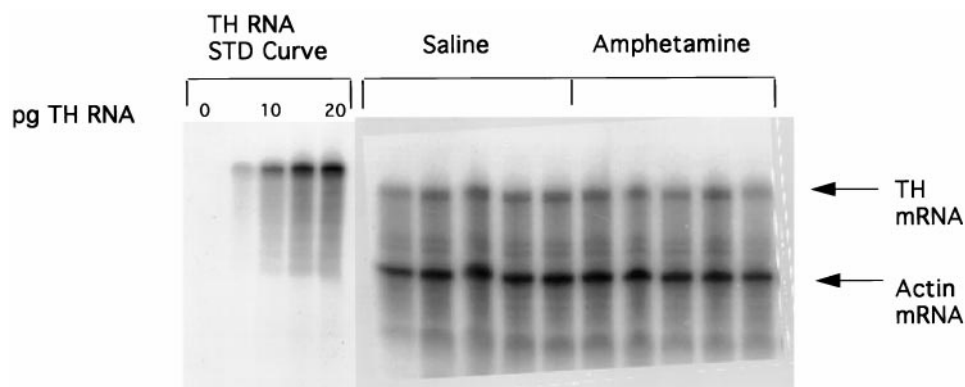
Data represent the mean \pm S.E. from the number of rats designated in the parentheses.

Fig. 4. Measurement of TH mRNA in midbrain samples from saline-treated and amphetamine-treated rats using RNase protection assays. Rats were treated with saline or amphetamine and total cellular RNA was isolated from the midbrain. This figure depicts data from the 4-month postamphetamine time point. The first five lanes of the figure depict a standard curve using known amounts (0, 5, 10, 15 and 20 pg) of sense strand TH RNA complementary to the antisense TH riboprobe used in the assay. These bands migrate more slowly on the gel compared with the sample TH mRNA duplex bands because they include restriction site sequences present within the multiple cloning region of the pGEM3. The remaining lanes depict assays using 10 μ g of midbrain RNA from saline-treated and amphetamine-treated rats as designated in the figure.

TABLE 3

Use of RNase protection assays to measure the effect of amphetamine on midbrain TH mRNA and actin mRNA levels

Rats were treated with saline or 3 mg/kg amphetamine four times once every 2 hr, and midbrains were isolated at the time points. TH mRNA and actin mRNA levels were measured using RNase protection assay and normalized to the micrograms of total cellular RNA put into the hybridization reactions.

| Treatment | Time after AMPH | TH mRNA | Actin mRNA | TH mRNA/actin mRNA |
|-----------|-----------------|------------------------|-----------------|--|
| | days | atmol / μ g of RNA | | atmol of TH mRNA / atmol of actin mRNA |
| Saline | 1 (5) | 0.14 \pm 0.02 | 0.74 \pm 0.08 | 0.20 \pm 0.02 |
| AMPH | 1 (5) | 0.16 \pm 0.02 | 0.79 \pm 0.05 | 0.20 \pm 0.02 |
| Saline | 3 (5) | 0.21 \pm 0.04 | 0.59 \pm 0.07 | 0.35 \pm 0.03 |
| AMPH | 3 (9) | 0.29 \pm 0.04 | 0.86 \pm 0.08 | 0.32 \pm 0.02 |
| Saline | 14 (4) | 0.27 \pm 0.02 | 0.80 \pm 0.07 | 0.34 \pm 0.02 |
| AMPH | 14 (6) | 0.28 \pm 0.06 | 0.80 \pm 0.07 | 0.38 \pm 0.03 |
| | months | | | |
| Saline | 4 (7) | 0.19 \pm 0.04 | 0.65 \pm 0.08 | 0.29 \pm 0.04 |
| AMPH | 4 (8) | 0.20 \pm 0.01 | 0.77 \pm 0.05 | 0.27 \pm 0.01 |

Data represent the mean \pm S.E. from the number of rats designated in the parentheses.

Effect of Amphetamine on TH Gene Expression in the Striatum

Although evidence was not found for the loss of TH-positive cell bodies in the midbrain after amphetamine treatment, the classic signs (Seiden and Sabol, 1995) of amphetamine neurotoxicity in the striatum were present at 14 days after amphetamine treatment. Extensive reactive gliosis was observed throughout the caudate/putamen, and TH immunoreactivity was greatly diminished in all areas of the striatum (data not shown). In addition, swollen TH-immunopositive fibers (axons) were seen throughout the caudate/putamen

and coursing through the globus pallidus (data not shown). At 4 months after amphetamine, areas in the medial to ventral lateral caudate/putamen, an area previously described by Eisch *et al.* (1992) to be most sensitive to methamphetamine, still showed a decreased number of TH immunopositive fibers and some swollen fibers.

Most notably, there also were TH-immunopositive cell bodies located within the caudate/putamen at 4 months after amphetamine (fig. 6). Many of these cell bodies appeared to have a cytoarchitecture that was similar to that of dopaminergic neurons in the substantia nigra; these were dorsome-

TABLE 4

Effect of amphetamine on midbrain TH protein levels

Rats were administered 3 mg/kg amphetamine four times once every 2 hr, and midbrains were removed at the times. Midbrain TH protein was measured using Western blot analysis and normalized to the micrograms of total cellular protein subjected to electrophoresis.

| Treatment | Time after amphetamine | Midbrain TH protein |
|-------------|------------------------|---|
| | days | $\mu\text{g}/\text{mg}$ of cellular protein |
| Saline | 1 | 0.43 ± 0.14 |
| Amphetamine | 1 | 0.49 ± 0.12 |
| Saline | 3 | 0.29 ± 0.03 |
| Amphetamine | 3 | 0.26 ± 0.08 |
| Saline | 14 | 0.34 ± 0.10 |
| Amphetamine | 14 | 0.26 ± 0.06 |
| | months | |
| Saline | 4 | 0.47 ± 0.14 |
| Amphetamine | 4 | 0.35 ± 0.10 |

Data represent the mean \pm S.E. from 5 or 6 rats.

No significant differences were calculated by ANOVA ($P > .05$) between values.

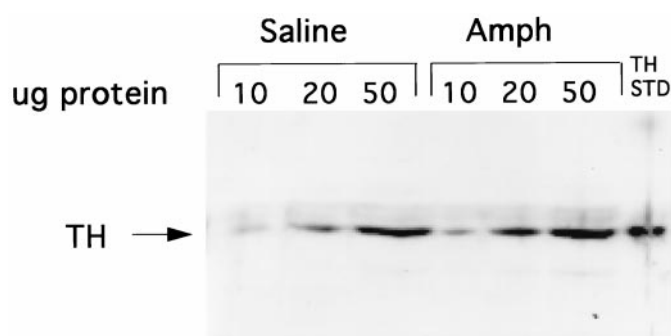


Fig. 5. Western analysis of TH protein in rat midbrain. TH protein was measured in the midbrain of saline-treated (lanes 1–3) and amphetamine-treated (lanes 4–6) rats. The autoradiogram depicts midbrain samples isolated from animals 14 days after amphetamine. For each sample three different concentrations (10, 20 and 50 μg as designated in the figure) of midbrain protein were loaded onto separate lanes of the gel. A known amount of purified TH protein was loaded onto lane 7 and was used to normalize signals between gels.

dially located in the caudate/putamen (fig. 6B). Other TH-positive cell bodies had a cytoarchitecture similar to either interneurons or multipolar neurons and were most often located laterally within 1 mm of the corpus callosum (fig. 6A). Almost all of the TH-positive cells were located in a narrow 1-mm band of the caudate/putamen defined anteriorly by the genu of the corpus callosum and posteriorly by the junction of the anterior commissures. A composite picture shows the location of these cells within the regions in which they occurred (fig. 7). These cells were not present in the medial caudate putamen and ventrally were only seen most laterally juxtaposed to the corpus callosum. The cells were present only in the 8 rats that had episodes of pronounced hyperthermia (body temperature of $>41^\circ\text{C}$) during amphetamine exposure and were most prominent in the 3 rats that had multiple episodes of pronounced hyperthermia. Striatal TH-positive cells were not detected in amphetamine-treated rats that did not become hyperthermic. Furthermore, TH-immunopositive cells were not observed in any of the age-matched controls or at 14 days after amphetamine. It was calculated that there were at most ~ 150 cells per hemisphere in the rats in which these cells were most conspicuous, because only 3 or 4 cells per hemisphere per section were visible.

Total cellular RNA from the striatum of saline- or amphet-

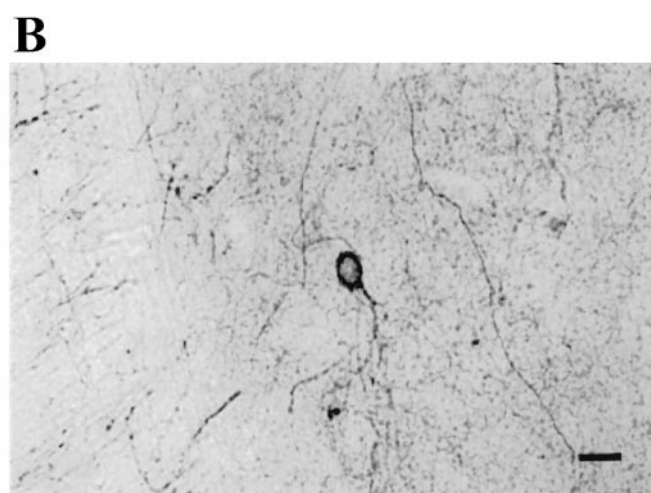
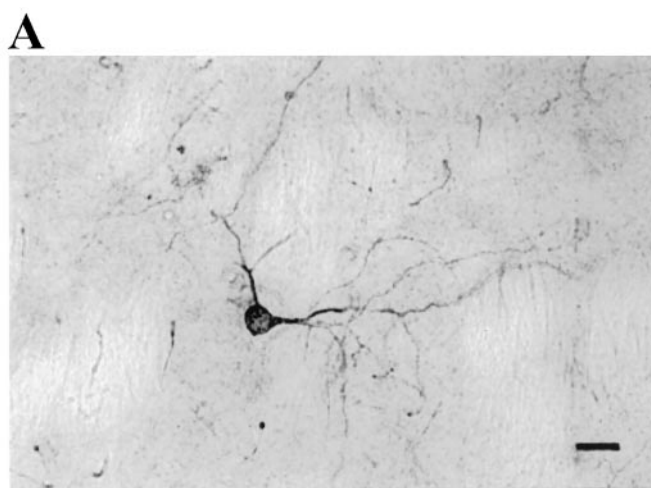


Fig. 6. Characteristics of TH immunopositive cells in the caudate/putamen 4 months after amphetamine exposure. A, TH-immunopositive soma and dendrites located in the lateral caudate/putamen (juxtaposed to the corpus callosum) of a coronal section taken $\sim +0.2$ AP to the bregma from an amphetamine-treated rat at 4 months after treatment. Bar, 20 μm . B, TH-immunopositive soma and dendrites located in the medial-lateral caudate/putamen of a coronal section taken $\sim +0.6$ AP to the bregma from an amphetamine-treated rat at 4 months after treatment. Bar, 20 μm .

amine-treated rats was analyzed to determine whether TH mRNA was present. Although RNase protection assays did not detect TH mRNA in the striatum of either saline- or amphetamine-treated animals, RT-PCR detected a significant level of TH mRNA in the striatum of all animals after a coupled 21 + 21 cycle PCR using different antisense 3' TH primers in the two steps of the coupled PCR (fig. 8). This RT-PCR signal from the striatum was not detectable using the PCR protocol used to detect TH mRNA in the midbrain. The levels of TH mRNA in the striatum were calculated to be at least 100 times lower than that observed in the midbrain. The RT-PCR data indicated that TH mRNA levels increased 2- to 3-fold in the amphetamine-treated rats ($n = 6$) that experienced pronounced hyperthermia (fig. 8). This increase in striatal TH mRNA levels was not observed in amphetamine-treated rats that did not become hyperthermic (data not shown).

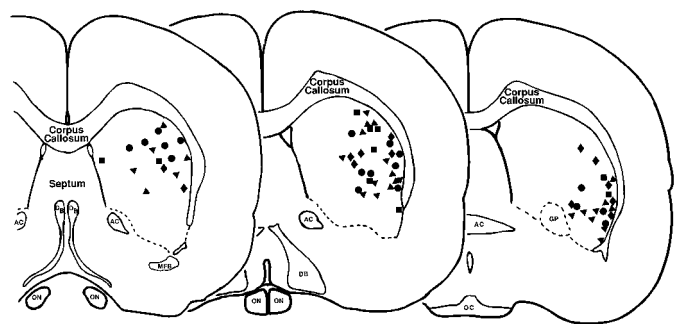


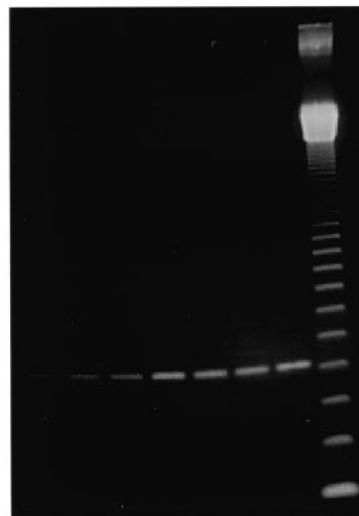
Fig. 7. A composite coronal section schematic of TH-immunopositive cells in the caudate/putamen 4 months after amphetamine. The figure depicts a composite schematic diagram of coronal sections compiled from five rats treated with amphetamine and killed at 4 months after amphetamine, showing the locations of the TH-immunopositive cells at the anterior (+0.80 from bregma), midpoint (+0.30 from bregma) and posterior (-0.20 from bregma) locations where such cells were found. Each type of symbol represents TH-positive cells observed in the same rat. Two sections within 60 μm of each of the three designated areas were evaluated for each rat to determine the number of cells exhibiting TH immunoreactivity. AC, anterior commissure; DB, diagonal band of Broca; GP, globus pallidus; MFB, medial forebrain bundle; OC, optic commissure; ON, optic nerve.

Discussion

Our results confirm previous findings that (1) amphetamine-induced decreases in striatal dopamine levels are associated with decreases in TH protein levels, suggesting that the persistent depletion of striatal dopamine is at least partially a consequence of the decrease in TH protein; and (2) even though amphetamine produces extensive damage that persists for months to dopaminergic axons and terminals in the striatum, there is no apparent loss of dopaminergic cell bodies in the midbrain. In addition, we show for the first time that (1) TH mRNA and TH protein levels in the midbrain cell bodies do not change, even transiently, after amphetamine exposure, indicating that TH gene expression is not induced in nigrostriatal neurons to compensate for the loss of dopamine in the terminal regions; and (2) amphetamine exposure triggers the expression of TH immunoreactivity in selective cells within the caudate/putamen, which is probably the result of induction of TH mRNA followed by a concomitant increase in TH protein synthesis. This phenotypic alteration in the striatum represents a novel type of neurotoxicity elicited by amphetamine.

The initial reports on the neurotoxicity of amphetamine and its derivatives described the depletion of dopamine and the degeneration of dopaminergic axons and terminals within the striatum (Seiden and Sabol, 1995). It was subsequently shown that in both rat and mouse, dopamine depletion, terminal degeneration and the following gliosis were greatly potentiated by the occurrence of significant hyperthermia during drug administration (Bowyer *et al.*, 1992, 1994; O'Callaghan and Miller, 1994). Although neuronal death also occurs in the parietal cortex (Commings and Seiden, 1986; Ryan *et al.*, 1990), as well as in the piriform cortex and hippocampal remnants (Schmued and Bowyer, 1997), neuronal cell loss is intermittent among these cortical neuronal populations and is not as pronounced as terminal loss in the striatum. Previous efforts to detect the loss of dopaminergic cell bodies in the midbrain after amphetamine or methamphetamine exposure have generally failed to observe any changes in the number of these cell bodies (Ricaurte *et*

A



B

Control Striatal RNA / Post AMPH Striatal RNA



Fig. 8. Ethidium bromide and phosphorimages of 519-bp RT-PCR products encoding TH cDNA from striatal total cellular RNA. Rats were treated with saline or amphetamine, and striata were isolated 4 months after treatment. Total cellular RNA was isolated from the striata and used in the RT-PCRs for measuring TH mRNA. A, Ethidium bromide staining of a 2% agarose gel containing RT-PCR products representing TH cDNAs produced using rat striatal RNA samples as templates. Each lane contained the same volume (15 μl) of a RT-PCR using striatal RNA and the specific 3'-927 TH primer for the RT step followed by 21 cycles of PCR coupled to another 21 cycles of PCR (5 μl of the first PCR was added to a second PCR mixture) as detailed in the text. Lanes 1 to 3 contained TH cDNA PCR products using striatal RNA from control animals, whereas lanes 4 to 7 contained TH PCR products using striatal RNA from amphetamine-treated rats 4 months after amphetamine. Lane 8 contained 3 μg of 123-bp ladder DNA standards. B, Phosphorimage of the radiolabeled RT-PCR products derived from the same reactions denoted in A.

al., 1982; Seiden and Sabol, 1995), although one group has recently reported dopaminergic cell body loss in this region after methamphetamine treatment in the mouse (Sonsalla *et al.*, 1996).

We attempted to optimize/maximize amphetamine neurotoxicity by using older animals and ensuring that they become hyperthermic during amphetamine exposure. All the results presented in this study are from rats that had body temperatures of $\geq 41^\circ\text{C}$ during amphetamine exposure. This paradigm produced dramatic depletion of striatal dopamine levels to 10% to 20% of control values at 3 to 14 days after amphetamine administration. Striatal TH protein levels also decreased by $\sim 65\%$ at the 14-day time point. Immunocytochemical analysis of the striatum showed dramatic loss of TH-positive terminals and axons at all time points tested, as

observed by numerous other workers (see review by Seiden and Sabol, 1995). Even 4 months after amphetamine treatment, striatal dopamine and TH protein levels remained significantly decreased. However, striatal dopamine levels recovered partially over this time to ~50% of that measured in control animals (compared with 10–20% of controls at the earlier time points). Hence, amphetamine treatment of older rats produced dramatic, long-term neurotoxic effects on the striatum. Yet, as reported by most others using other amphetamine derivatives and administration paradigms (Ricaurte *et al.*, 1982; Ryan *et al.*, 1990; O'Callaghan and Miller, 1994), in which dopamine depletions were not as pronounced, there was no detectable loss of TH-positive neurons in the rat midbrain. In addition, the observation that striatal TH protein levels did not decrease to the same extent as dopamine levels at the early time points suggests that other factors governing the steady-state levels of striatal dopamine, such as reuptake transporter systems, storage vesicles and/or metabolizing enzymes, may also be affected by amphetamine.

This lack of apparent neurodegenerative effects on dopaminergic cell bodies in the midbrain coupled with the partial recovery of dopamine levels in the striatum at 4 months after drug treatment led us to hypothesize that TH gene expression may be altered in the cell bodies to maintain an appropriate concentration of TH protein in the surviving striatal terminals over time. Induction of TH mRNA and TH protein occurs peripherally in the adrenal medulla and sympathetic ganglia after environmental stress or treatment with catecholamine-depleting drugs, like reserpine or 6-hydroxydopamine (Zigmond *et al.*, 1989b; Kumer and Vrana, 1996). Presumably, this induction occurs to compensate for the loss of catecholamines from nerve terminals due to pronounced exocytosis or nerve terminal damage. Induction of TH within the central nervous system has also been reported in noradrenergic neurons of the locus coeruleus, but it has been difficult to detect regulation of TH gene expression within dopaminergic neurons in substantia nigra (Kumer and Vrana, 1996). In a paradigm similar to our amphetamine neurotoxicity studies, Pasinetti *et al.* (1989, 1992) used 6-hydroxydopamine to produce partial lesions of nigrostriatal dopamine neurons (by ~60%). This partial lesion produced a transient decrease in striatal dopamine levels at 21 days after treatment; however, striatal DA levels recovered back to control levels by 90 days after treatment. Similarly, striatal TH enzyme activity and protein levels decreased transiently at 21 days but returned to control levels by 90 or 270 days after partial lesioning. In contrast, TH mRNA levels per surviving cell body in the substantia nigra did not change when measured 2 or 21 days after treatment and decreased by ~30% and ~50% at 90 days and 270 days after lesioning, respectively. 6-Hydroxydopamine produces cell body death in the midbrain as well as terminal damage in the striatum. In contrast, amphetamine produces only damage to the striatal nerve terminals. Hence, we hypothesized that TH mRNA and TH protein in the undamaged midbrain cell bodies may be induced to compensate for the loss of dopamine at the nerve terminals.

In the present study, amphetamine treatment produced a greater depletion of striatal dopamine (80–85%) than occurred in the 6-hydroxydopamine study. Nevertheless, midbrain TH mRNA levels did not change at any time point after amphetamine treatment. Blanchard *et al.* (1995) recently

reported similar results using 6-hydroxydopamine to produce 30% to 70% lesions of nigrostriatal neurons, in which TH protein and TH mRNA levels in nigral dopaminergic cell bodies did not change at 30 days after these lesions. However, in contrast to our studies and the earlier reports of Pasinetti *et al.*, this more recent study reports an induction of TH mRNA in nigral cell bodies 6 months after lesioning. However, this induction is very small (20–30%) and it is not clear whether it is physiologically meaningful. Even though the results of the partial lesion studies using 6-hydroxydopamine are not fully conclusive, the most striking result is that in agreement with the results in the present study using neurotoxic doses of amphetamine, TH mRNA and TH protein are not induced dramatically in surviving midbrain cell bodies. This lack of compensatory induction of TH mRNA after lesioning of striatal dopaminergic nerve terminals by either 6-hydroxydopamine or amphetamine is puzzling. One would anticipate the existence of robust compensatory homeostatic mechanisms to elevate TH gene expression to help increase dopamine biosynthesis in surviving striatal nerve terminals and midbrain cell bodies and to maintain high levels of TH protein in newly sprouted striatal nerve terminals. It is possible that excitatory input to the midbrain, which would be expected to induce TH gene expression, is prevented during these lesioning paradigms or that inhibitory input into the midbrain prevents the appropriate up-regulation of the TH gene. Further work is needed to test these hypotheses.

Perhaps the most intriguing aspect of the present study is the observed generation of TH immunopositive neurons and the detection of TH mRNA in the striatum 4 months after amphetamine treatment (figs. 6–8). TH-positive neurons are not detected in control rats or in rats killed 4 months after amphetamine treatment but that do not become hyperthermic during drug administration. Furthermore, we have never observed the appearance of TH-immunopositive neurons in the striatum of control or amphetamine-treated rats at 2 weeks or less after exposure (Bowyer *et al.*, 1994; unpublished data). Additional studies are necessary to determine whether TH-positive neurons are generated by amphetamine exposure in younger rats (<15 months of age).

The finding of these TH-positive cell bodies in the striatum of amphetamine-treated rats is supported by the observed increases in the levels of striatal TH mRNA in these rats. Striatal TH mRNA is not detected using RNase protection assays; however, the extremely sensitive RT-PCR assay detects a 519-bp PCR product, which is the expected product encoding TH cDNA. This PCR product is detectable in both control and amphetamine-treated rat striatum. The detection of this TH PCR product in control animals suggests that small amounts of TH mRNA may be expressed promiscuously in striatal cells. Pertinent to this study, the most notable finding is that the formation of this product reproducibly increases when striatal RNA from amphetamine-treated rats that became hyperthermic is used in the RT-PCR. This increase is consistent with the observed generation of a small number of TH-positive cells in the striatum after amphetamine treatment and suggests that the expression of TH immunoreactivity in these cells is a consequence of increased TH gene expression. It should also be noted that striatal TH mRNA levels were not increased in amphetamine-treated rats that did not become hyperthermic; hence, the neurotoxicity associated with amphetamine-elicited hyperthermia is

apparently required for the production of these striatal TH-positive cells.

Interestingly, TH-positive cells are not observed in the medial portion of the caudate/putamen. Dopamine levels are not as depleted and recover more rapidly in this region compared with other regions of the caudate/putamen (Eisch *et al.*, 1992; J. F. Bowyer, P. Clausing and R. R. Holson). It is possible that this is because this area receives dopaminergic input from the ventral tegmental area; these dopaminergic neurons are resistant to toxic insult relative to nigrostriatal neurons (German *et al.*, 1992). However, because few TH-positive cells are generated in the ventral-medial caudate/putamen, an area greatly depleted of dopaminergic innervation after amphetamine or methamphetamine treatment, the magnitude of the loss of dopaminergic input is not sufficient to explain the generation of these TH-positive cells.

It is likely that neurotrophic factors or growth factors play a role in the initiation of TH gene expression in previously noncatecholaminergic striatal cells. Du and Iacovitti (1997) have shown that the treatment of cultured striatal neurons (which do not express TH under basal culture conditions) with a number of different neurotrophic factors in combination with a partner molecule, possibly an activator of protein kinase C, is sufficient to induce quiescent TH gene expression. These or other factors could be involved in the generation of TH-expressing neurons in the striatum *in vivo* after treatment with amphetamines. Alternatively, progenitor cells from the rostral midbrain are another possible source for the TH-positive cells in the striatum 4 months after amphetamine (Reynolds and Weiss, 1992). These cells are still found in adult animals and can be manipulated *in vitro* via cytokines and neurotrophic factors to produce TH and a partial conversion to a dopaminergic phenotype (Ptak *et al.*, 1995). However, whether there is a sufficient number of progenitor cells in 15-month-old rats and whether they can migrate the distances in the brain necessary to reside in the medial/anterior striatum remain obscure.

The results of these studies and others (Seiden and Sabol, 1995) showing that methamphetamine and amphetamine damage axons and terminals and not the soma of dopaminergic neurons may explain the lack of human clinical evidence correlating amphetamine abuser populations with Parkinson's disease-like symptoms or movement disorders. Because there is a slow recovery in striatal dopamine levels after methamphetamine or amphetamine exposure, this would tend to ameliorate the initial neurotoxicity. The recovery of dopamine levels in the striatum after amphetamine appears to be primarily due to regeneration of axons and terminals. However, our results show that this recovery probably is not associated with a compensatory up-regulation of TH gene expression in the midbrain cell bodies. Furthermore, in humans, the primary effect of methamphetamine abuse in the striatum may be limited to regulatory changes in dopaminergic terminals and not terminal/axonal degeneration (Wilson *et al.*, 1996). Even if terminal degeneration occurs in humans after exposure to high levels of amphetamine or methamphetamine, it may be restricted to only those who experience significant hyperthermia because in rodent models, particularly the rat, hyperthermia is an essential factor in generating dopaminergic axonal/terminal damage (Bowyer *et al.*, 1992, 1994; O'Callaghan and Miller, 1994).

Acknowledgments

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