

## Research report

# Tissue-specific glucocorticoid regulation of tryptophan hydroxylase mRNA levels

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Accepted 25 February 1997

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**Abstract**

A potential long-term target of glucocorticoid modulation of serotonin (5-HT) production is tryptophan hydroxylase (TPH) gene expression. However, studies on TPH gene expression have been hampered by the extremely low levels of TPH mRNA in the brain, and there have been contradictory reports on the effects of glucocorticoids on 5-HT levels. To overcome these obstacles, we have developed a sensitive competitive RT-PCR assay to directly measure TPH mRNA levels from the rat brain. We observed a tissue-specific modulation of TPH mRNA levels in the melatonin producing pineal gland and the serotonin producing raphe nuclei of the brain. Following chronic treatment of adrenalectomized rats with the synthetic glucocorticoid dexamethasone for 1 week, there was a 16-fold increase in TPH mRNA in the pineal gland that was contrasted by a decrease in TPH mRNA to 16% of the control levels in the brain. To address the mechanism of dexamethasone repression of TPH mRNA levels, we then tested a serotonergic neuronal-like cell line derived from rat thyroid C cells. Dexamethasone caused a rapid decrease in TPH mRNA levels to  $\approx 20\%$  of control values in CA77 C cells. This was measured by both competitive RT-PCR and a standard hybridization assay, which confirmed the validity of the RT-PCR assay. Furthermore, the reduction of TPH mRNA levels was associated with a decrease in 5-HT levels in the CA77 C cells. Hence, glucocorticoids may alter serotonin and melatonin biosynthetic capacity by cell-specific modulation of the TPH gene.

**Keywords:** Glucocorticoid; Serotonin; Melatonin; Tryptophan hydroxylase; Thyroid C cell; Parafollicular cell; Competitive RT-PCR

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**1. Introduction**

Glucocorticoids have pleiotropic effects in mammals. They are widely recognized to alter mood and sleep, particularly after prolonged administration at high doses [24,30,39]. Similar symptoms may also be observed in patients with Cushing's syndrome [30]. An important mediator of these biological functions and pathologies is the neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) [3,6,25], and the hormone melatonin may also contribute to sleep disorders [1,16,17,35]. A common biosynthetic step shared by these neurochemicals involves the enzyme tryptophan hydroxylase (TPH), which is the rate limiting enzyme for 5-HT biosynthesis in the raphe nuclei of the brain [21], and is essential for biosynthesis of melatonin in the pineal gland [29]. Hence, a reasonable target for gluco-

corticoid action on serotonergic function, as well as melatonin production, is TPH.

Despite interest in 5-HT and melatonin and their relationships to glucocorticoids in affective and sleep disorders, there have been conflicting reports on the effects of glucocorticoids on these neurochemicals. There have been no reports on glucocorticoid regulation of pineal TPH mRNA or protein levels and effects on melatonin release are contradictory; with increases, decreases, and no effect all, having been reported [27,31,47,50]. In the case of the raphe nuclei, short-term, but not long-term effects of glucocorticoids on TPH have been reported. Glucocorticoid treatment for 1–3 days produces a 1.8-fold increase in TPH immunoreactivity [2], and acute sound stress increases TPH activity in a glucocorticoid-dependent manner [41,42]. However, short- and long-term glucocorticoid treatment may not produce identical biological results [14,36] and, unfortunately, contradictory effects of chronic glucocorticoid treatment on 5-HT levels have been re-

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ported [8]. These discrepancies could be due to the large number of variables, such as those that occur during tissue collection and similar experimental manipulations, that may acutely modulate the absolute and relative levels of intracellular and extracellular 5-HT. Studies on TPH protein are complicated by the instability of TPH protein in vitro [26], and the cross-reactivity of most available TPH antibodies [2].

In this report, we have tested the hypothesis that chronic glucocorticoid treatment regulates TPH mRNA levels. We have opted to examine the effects of chronic exposure since chronically elevated glucocorticoid levels are most associated with mood and sleep disturbances. TPH mRNA levels were measured since this is a reasonable target for long-term regulation of 5-HT, as well as melatonin, biosynthetic potential. However, TPH mRNA is a rare transcript in the serotonergic raphe nuclei of the brainstem, and is extremely difficult to detect in this tissue by conventional methods, such as Northern blots [15,18]. Even using ribonuclease protection assays, detection of TPH mRNA requires pooling of 5–10 dissected brainstems [18,23]. Thus, we have chosen to use competitive RT-PCR, an accurate method of measuring rare RNA transcripts [11,13,19,20,40,48,49]. We demonstrate that the synthetic glucocorticoid dexamethasone can lower TPH mRNA levels in the raphe nuclei, while increasing the levels in the pineal gland. This suggests a change in the biosynthetic capacity of cells to produce serotonin and melatonin. To test this prediction we used the serotonergic neuronal-like CA77 cell line since 5-HT levels in a cell line are not as susceptible to the large number of factors that may have confounded past measurements of 5-HT in vivo. These results suggest that TPH biosynthetic capacity may be regulated in vivo by chronic exposure to high levels of glucocorticoids.

## 2. Materials and methods

### 2.1. Cell culture and animal care

CA77 cells were maintained as previously described [37]. Adrenalectomized 200 g male Sprague-Dawley rats (Harlan, Chicago, IL) were given either 2.5 mg dexamethasone sodium phosphate (Elkins-Sinn, Cherry Hill, NJ) or saline vehicle by s.c. injection once per day for 7 days. On the 8th day, a final injection was given 4 h before the rats were euthanized by CO<sub>2</sub> asphyxiation or halothane inhalation. The brain and loosely associated pineal gland were rapidly removed at the level of the C1 vertebrae. Poly(A)-enriched RNA was prepared from the brain and CA77 cells by proteinase K-oligo (dT) chromatography as described [37]. Total RNA was prepared from pineal glands by the acid-guanidinium-isothiocyanate method as described [9]. For studies involving 1 day of treatment,

adrenalectomized rats were held for 6 days without treatment, then treated on the 7th and 8th days as above. All rats were maintained on 0.9% saline, rat chow ad lib, and a 12 h light/dark cycle. All injections and euthanasia were performed during light periods. All animals were handled in accordance with state and federal regulations.

### 2.2. Competitive RT-PCR constructs and RNA synthesis

Plasmid pTPH400 contains a 396 bp TPH cDNA fragment (bases 942–1338 of rat TPH cDNA) in the pGEM-T vector [9]. The plasmid used to synthesize TPH competitor RNA, pTPH-Ad1, was constructed by inserting a 225 bp fragment of adenovirus intron DNA into the internal *Hind*III site of pTPH400. When amplified with the same primers used to generate the original 396 bp TPH fragment, pTPH-Ad1 produces a 621 bp product. In order to produce competitor and control TPH RNA, pTPH400 and pTPH-Ad1 were linearized at a *Spe*I site contained within the pGEM-T polylinker, and transcribed in vitro using T7 RNA polymerase as described [11]. RNA yield was quantified by incorporation of tracer amounts of  $\alpha$ -<sup>32</sup>P-UTP (Amersham) during in vitro transcription. Plasmid DNA was removed from the reactions using RNase-free DNase (Promega, Madison, WI).

### 2.3. Competitive RT-PCR and filter hybridizations

RT and PCR reactions were performed essentially as described [9,12]. Briefly, aliquots of sample RNA were mixed with known quantities of competitor RNA, and reverse-transcribed using a primer corresponding to bases 1323–1338 of rat TPH cDNA (5'-TTCACACACTGG-GCCA-3'). A 1  $\mu$ l aliquot of each RT reaction was amplified in a 50  $\mu$ l reaction containing 20 mM Tris-HCl, pH 8.6, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 30  $\mu$ g/ml bovine serum albumin, 200  $\mu$ M deoxynucleotides, 0.5  $\mu$ M each of the downstream primer (bases 1323–1338) and upstream primer (bases 942–971) (5'-TTCAGTGTGGAGTT-TGGACTGTGCAAGCAA-3'), and 1.25 U *Taq* polymerase (Boehringer Mannheim, Indianapolis, IN). The reactions were amplified for 30 cycles at 94°C for 1.5 min, 55°C for 1 min, 72°C for 2 min, and a final 5 min incubation at 72°C. For some experiments, *Taq* polymerase was added only after heating reactions mixtures to 65°C. Amplification products were separated by electrophoresis on 1.2% to 1.5% agarose gels and visualized by ethidium bromide staining. Southern and Northern blots and hybridizations were performed as described [9,12].

### 2.4. Competitive RT-PCR data analysis

Densitometric analysis was performed using NIH Image v1.58 software on scanned high resolution images of gel photos or autoradiographic films. The ratio of competitor

band density to TPH band density within individual reactions was determined using bundled NIH Image macros. Equivalency points were calculated as described [20]. Briefly, for each experiment, the log of the competitor/TPH band density ratios (*Y*-axis) was plotted vs. the log of competitor TPH mRNA attomolar concentration (*X*-axis). The *X*-intercept is where the log of the competitor/TPH band density ratio equals 0, and by definition is the equivalency point. Based on pilot measurements, the experiments were designed so that the equivalency point was near one of the assay points for the most accurate determination. In some experiments, weaker bands were observed. The one slightly below the competitor band is probably a heteroduplex of competitor and TPH. This band was routinely omitted from the calculations since inclusion of it with either the competitor or TPH signals did not qualitatively change the results. A diffuse band was observed in many samples beneath the TPH band. This band was not included in the calculations. It most likely represents primer-dimer products since it was present even in the absence of competitor and TPH RNA. In addition, its intensity decreased with increasing TPH and/or competitor products, further suggesting that it was derived from primers rather than RNA.

## 2.5. 5-HT and 5-HIAA determinations

Both 5-HT and 5-HIAA (5-hydroxyindole acetic acid) in cultured CA77 cells were measured by high performance liquid chromatography (HPLC) with electrochemical detection as described [43]. The cells were grown in

serum-free ITS medium [37] in 100 mm dishes to  $\approx 5 \times 10^6$  cells per control dish and  $2 \times 10^6$  cells per dexamethasone dish, then rapidly lysed with 1 ml of ice-cold 0.1 N perchloric acid, 1 mM ascorbic acid. Measurements were made from five dishes each of control and dexamethasone-treated cells. Recovery was monitored and corrected by addition of DHBA, although this did not alter the values by  $> 5\%$ . The raw data values for 5-HT were  $95.0 \text{ pg} \pm 6.6$  (S.E.M.) per  $20 \mu\text{l}$  of control cell lysate (limit of detection 5–10 pg per  $20 \mu\text{l}$ ). The number of cells per dish was determined using a hemocytometer.

## 3. Results

### 3.1. Establishment of a competitive RT-PCR assay for TPH mRNA

The first objective was to establish a competitive RT-PCR assay for TPH mRNA. Competitive RT-PCR utilizes *in vitro* transcribed RNA as an internal standard (the competitor) that is added in known quantities to a series of RT-PCR reactions. The competitor uses the same primers as the transcript to be measured (the target), but produces a different length product that can be distinguished from the target product. Because the two products ‘compete’ for the same primers and reagents, reaction efficiency is internally controlled. Thus, when the molar competitor to target ratio of the amplification products is equal (the equivalency point), the initial concentrations of each RNA species are also equal [11,13,19,20,40,48,49]. We have calculated

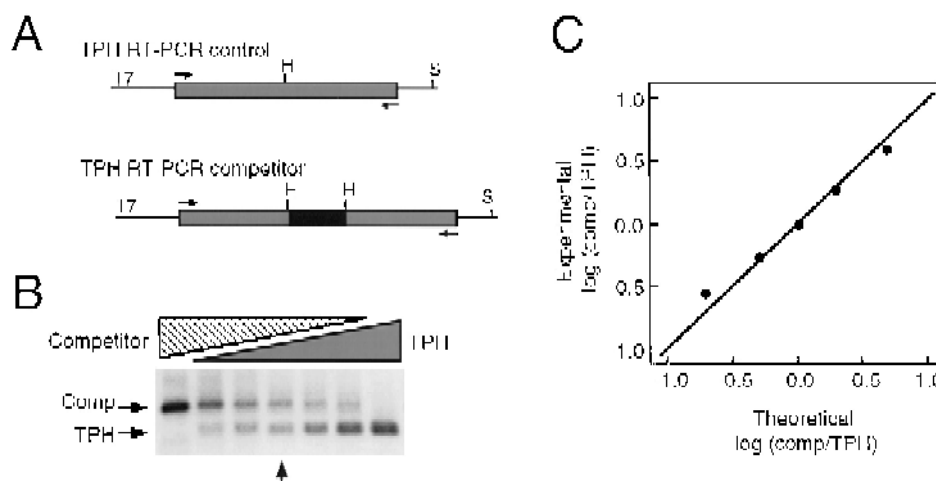


Fig. 1. Establishment of competitive RT-PCR for TPH RNA measurement. A: a TPH PCR product was cloned into pGEM-T to produce a TPH control plasmid. A 225 bp fragment of DNA (indicated in black) was inserted into an internal *Hind*III (H) site to produce the TPH competitor plasmid. The *Spe*I (S) site used to linearize the plasmid prior to *in vitro* transcription is indicated. Locations of primers for RT-PCR are indicated by arrows. B: *in vitro* synthesized control and competitor RNAs were mixed in varying molar ratios (competitor to TPH ratios, left to right: 6:0, 5:1, 4:2, 3:3, 2:4, 1:5, 0:6) reverse-transcribed, and amplified by PCR. As indicated by an arrow, the ratio of competitor to control TPH mRNA RT-PCR products is equal at the point of equal molar concentrations of the starting RNAs. Products were detected by ethidium bromide staining; a negative image is shown. C: to assess the accuracy of the assay, the competitor and TPH band densities were quantified using NIH Image, and the competitor to TPH ratio determined. Since the starting concentrations of both competitor and TPH were known, the experimental data (indicated by the circles) was compared to the predicted ratios (indicated by the line). Data obtained near the equivalency point (where the log ratio is 0) are in good agreement with predicted values.

equivalency points by the method of Gilliland et al. [20]; using a double-log plot of the ratio of competitor to target signals vs. the amount of TPH competitor in each reaction. The TPH competitor concentration at which the log of the competitor to target ratio is 0 is, by definition, the equivalency point. A strength of this methodology is that the equivalency point is calculated from a standard curve generated from multiple data points, rather than from extrapolation of the ratio from a single point. Furthermore, because of the assay sensitivity, multiple animals can be independently assayed. Another key feature is that it is the ratio of the competitor to target within a single reaction tube that is the critical parameter in this assay and not absolute differences in TPH or competitor band intensities, which can vary due to different reaction efficiencies between tubes.

In order to produce a competitor for TPH, a fragment of heterologous DNA was inserted into a rat TPH cDNA clone (Fig. 1A). Competitor RNA for reverse transcription was synthesized *in vitro* and known quantities of TPH RNA were similarly produced. Competitor and TPH RNAs were mixed in varying molar ratios, reverse-transcribed

and amplified by PCR. As predicted, when initial molar ratios of RNA were equal, molar ratios of the RT-PCR products were equal (Fig. 1B). Furthermore, the assay appeared to produce results essentially identical to the theoretical prediction (Fig. 1C). In addition, a repeated measurement of the same RNA sample gave a standard deviation of < 12%.

### 3.2. Dexamethasone increases TPH mRNA levels in the pineal gland

To assess the effects of chronic glucocorticoid treatment on TPH mRNA levels in the pineal gland, adrenalectomized male rats were given daily injections of dexamethasone or vehicle alone for 7 days. Adrenalectomized animals were used to avoid confounding effects of endogenous glucocorticoids and to maximize our ability to detect dexamethasone induced changes in TPH mRNA levels. RNA was isolated from the pineal gland and analyzed for TPH mRNA content by competitive RT-PCR (Fig. 2A). Mean TPH mRNA levels in the pineal of vehicle-treated animals was 112 amol TPH mRNA/ $\mu$ g total RNA, while

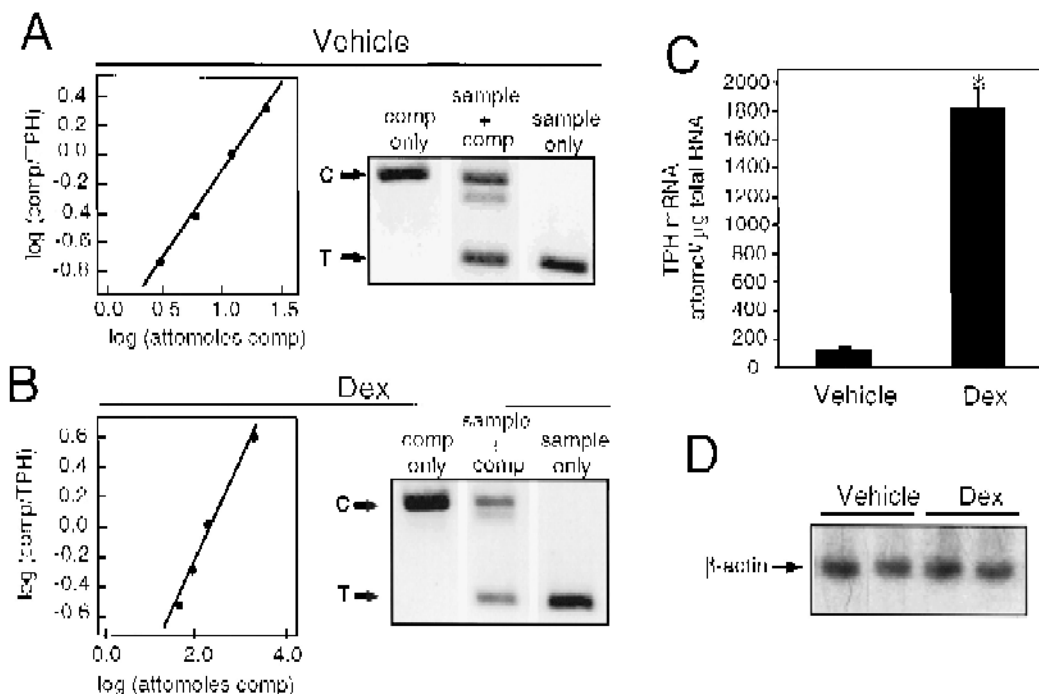


Fig. 2. Dexamethasone increases TPH mRNA levels in the pineal gland. TPH mRNA content was measured in samples of pineal total RNA (0.125  $\mu$ g) by competitive RT-PCR. Competitor and TPH band densities were quantified using NIH Image, and the competitor to TPH ratio determined for a range of competitor concentrations. The ratio was plotted against the number of amol of competitor in a double-log plot. Best-fit lines were plotted using linear regression analysis. TPH mRNA concentration in each sample was equivalent to competitor RNA concentration where the log competitor/TPH ratio equaled 0. Examples of the RT-PCR results are shown, with lanes corresponding to competitor only, sample and competitor near equivalency, and sample only, as indicated (C, competitor; T, TPH). Products were examined using ethidium bromide staining with negative images shown. amol =  $10^{-18}$  mol. A: RNA from vehicle-treated adrenalectomized rats. B: RNA from dexamethasone-treated adrenalectomized rats. C: mean TPH mRNA levels measured by competitive RT-PCR were determined for both vehicle-treated ( $112 \pm 28$  amol TPH mRNA/ $\mu$ g total RNA,  $n = 3$ ), and dexamethasone-treated animals ( $1800 \pm 145$  amol TPH mRNA/ $\mu$ g total RNA,  $n = 2$ ). TPH mRNA values were normalized to  $\beta$ -actin levels determined by Northern blots. Error bars indicate S.E.M. (Student's *t*-test, two-tailed, \*  $P = 0.035$ ). D: integrity and concentration of RNA was confirmed by a Northern blot probed for  $\beta$ -actin. Representative blots from rats given either the vehicle or dexamethasone are shown.

that in dexamethasone-treated animals was 1800 amol TPH mRNA/ $\mu$ g total RNA (Fig. 2B). Hence, in the pineal gland, dexamethasone treatment increased TPH mRNA levels by 16-fold (Fig. 2B). Essentially identical results were obtained following 1 day of dexamethasone treatment as measured by RT-PCR (data not shown). In order to confirm RNA integrity and quantification, relative  $\beta$ -actin RNA levels were measured by Northern blot (Fig. 2C). Thus, dexamethasone increases TPH mRNA levels in the pineal gland of adrenalectomized rats.

### 3.3. Dexamethasone decreases TPH mRNA levels in the raphe nuclei

We then determined the long-term effect of glucocorticoids on the serotonergic neurons of the raphe nuclei. Since TPH mRNA within the brain is present only within the raphe nuclei of the brainstem [45], separation of the loosely associated pineal gland from the remainder of the brain allowed us to analyze the two regions independently by a simple and reproducible dissection. While a more selective dissection would have increased the relative RNA contribution from the raphe, it would also have increased the potential for error due to differences in non-raphe RNA

content, and would still have required the RT-PCR assay. RNA was isolated from same rats used for the pineal TPH measurements after treatment with dexamethasone for 7 days. TPH mRNA levels in each RNA sample were determined by competitive RT-PCR (Fig. 3A). The mean TPH mRNA level in vehicle-treated animals was 8.26 amol TPH mRNA/ $\mu$ g A<sup>+</sup> RNA; while that for dexamethasone-treated animals was 1.35 amol TPH mRNA/ $\mu$ g A<sup>+</sup> RNA (Fig. 3B). Hence, chronic dexamethasone treatment reduced TPH mRNA levels by 6.1-fold. In order to confirm RNA integrity and quantification, relative  $\beta$ -actin RNA levels were measured by Northern blot (Fig. 3C). In contrast to results with long-term glucocorticoid treatment, administration of dexamethasone for 1 day resulted in a slight (< 2-fold), statistically insignificant, increase in TPH mRNA levels over vehicle-treated animals (data not shown). While further experiments are needed to address this point, this observation is consistent with the results of Azmitia et al. [2].

These findings demonstrate that 1 week of dexamethasone treatment produces an increase in TPH mRNA levels in the pineal gland, while the same glucocorticoid treatment reduces TPH mRNA levels in the raphe. Thus, glucocorticoids appear to regulate TPH mRNA levels in a tissue-specific manner in the adrenalectomized male rat.

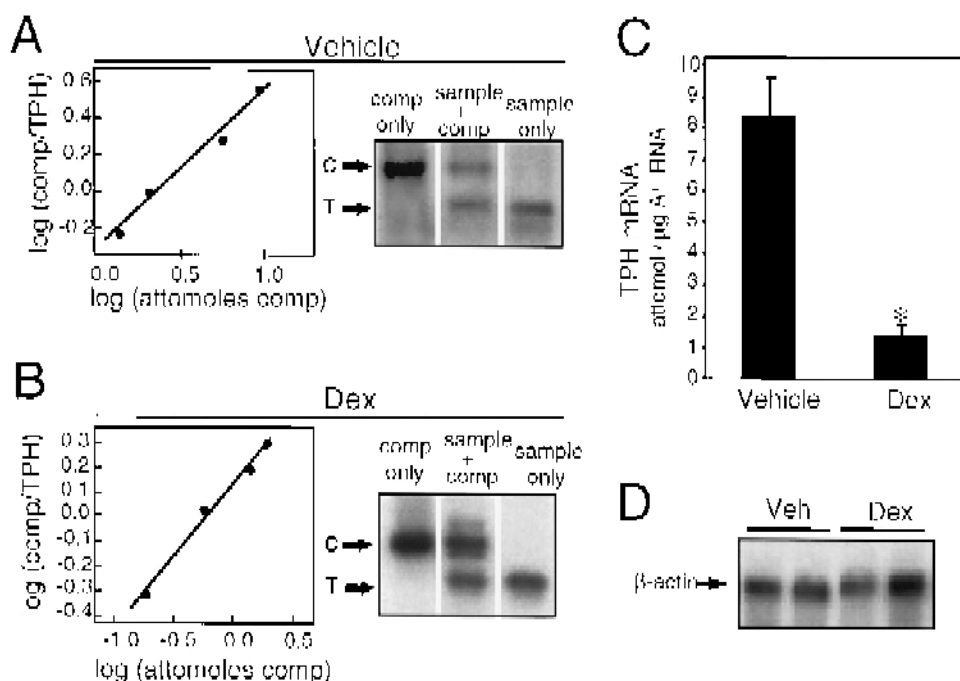


Fig. 3. Chronic dexamethasone treatment represses TPH mRNA levels in the brain. A: TPH mRNA content was measured in brain A<sup>+</sup> RNA (0.25  $\mu$ g) from vehicle-treated adrenalectomized rats by competitive RT-PCR. Competitor and TPH band densities were measured and plotted as described in Fig. 2. Sample RT-PCR results are shown, with lanes corresponding to competitor only, competitor and sample near equivalency, and sample only. Products were examined by Southern blots. B: same as A, except RNA was from dexamethasone-treated adrenalectomized rats. C: mean TPH RNA levels measured by competitive RT-PCR were determined for both vehicle-treated ( $8.26 \pm 3.2$  amol TPH mRNA/ $\mu$ g A<sup>+</sup> RNA,  $n = 6$ ), and dexamethasone-treated ( $1.35 \pm 0.89$  amol TPH mRNA/ $\mu$ g A<sup>+</sup> RNA,  $n = 6$ ) animals. TPH mRNA values were normalized to  $\beta$ -actin levels determined by Northern blots. Error bars indicate S.E.M. (Student's  $t$ -test, two-tailed, paired, \*  $P = 0.0052$ ). D: integrity and concentration of brain RNA was confirmed by a Northern blot probed for  $\beta$ -actin, with representative samples from vehicle- and dexamethasone-treated rats shown.

### 3.4. Dexamethasone decreases TPH mRNA levels in the CA77 cell line

In order to further assure the validity of the competitive RT-PCR assay and begin to address the mechanism of cell-specific regulation, we switched from the heterogeneous brain tissue to a homogenous CA77 cell line derived from thyroid C cells. Thyroid C cells (also called parafollicular cells) express TPH, synthesize and secrete serotonin, and possess 5-HT<sub>1B</sub> autoreceptors [5,9,32]. C cell lines, as well as NGF-treated C cells, also extend neurites and express neuronal 5-HT transporter and monoamine oxidase A [4,9,10,37,46]. As CA77 cells have been shown to produce TPH mRNA in levels detectable by Northern hybridization [10], they are especially useful since measurements by conventional RNA slot-blot analysis can be directly compared to competitive RT-PCR results.

CA77 cells were treated with dexamethasone or vehicle alone for 5 days. At drug concentrations of 10 nM or greater, TPH mRNA levels were reduced by 5–6-fold, as determined by RNA slot-blot (Fig. 4A,B). The dose re-

sponse of the repression is consistent with the 3–7 nM *K<sub>d</sub>* of the glucocorticoid receptor for dexamethasone (Fig. 4B) [34]. Repression was also fairly rapid, with maximal effects seen within 24 h (Fig. 4A). As a confirmation of RNA integrity and quantification, the same slot-blots were stripped, allowed to decay to background, and probed for  $\beta$ -actin (Fig. 4A). When the same RNA was analyzed by competitive RT-PCR, a similar 5-fold reduction in TPH mRNA levels was measured (Fig. 5). This supports the equivalent accuracy of the two methodologies in establishing relative TPH RNA levels. Furthermore, as discussed below, these results suggest that glucocorticoids have the potential to directly act on serotonergic cells to decrease TPH mRNA levels.

### 3.5. Dexamethasone decreases 5-HT / 5-HIAA levels in the CA77 cell line

In order to determine whether the dexamethasone induced decrease in TPH mRNA levels would lead to a decrease in 5-HT levels, CA77 cells were treated with

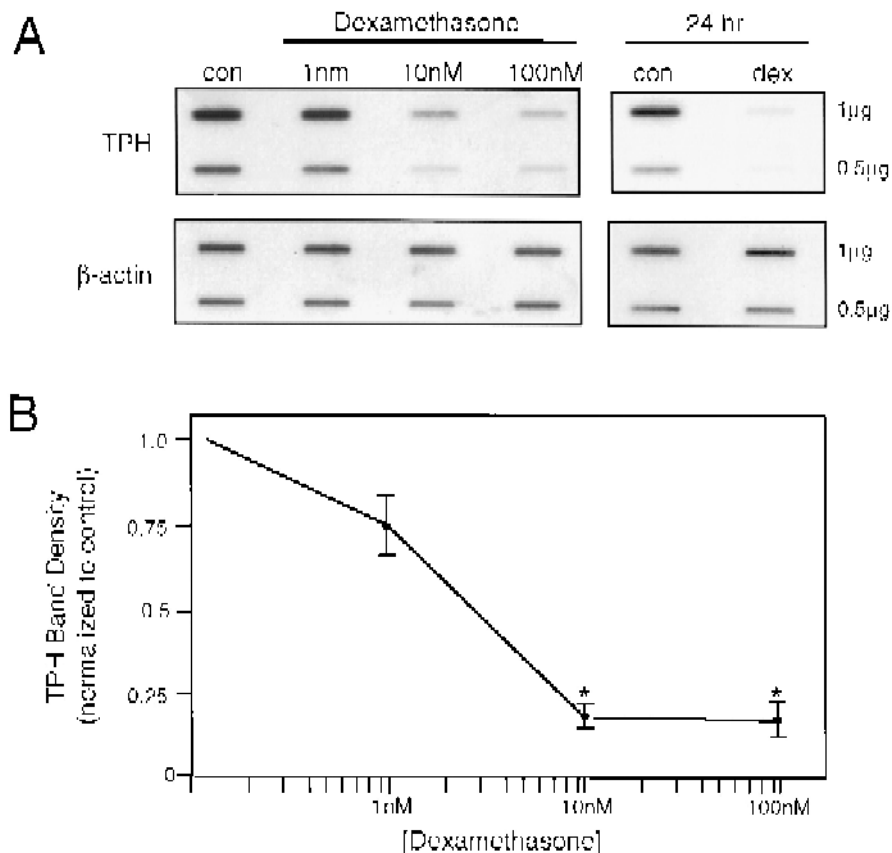


Fig. 4. Dexamethasone represses TPH RNA levels in CA77 thyroid C cells. A: CA77 cells were treated either with vehicle alone (con) or varying concentrations of dexamethasone (dex) for 5 days; or vehicle alone or 500 nM dexamethasone for 24 h, as indicated. A<sup>+</sup> RNA (0.5 or 1  $\mu$ g) was isolated and analyzed for TPH RNA by slot-blot hybridization. The same filters were stripped, allowed to decay to background, and re-probed for  $\beta$ -actin. B: TPH band intensities were determined, normalized to  $\beta$ -actin, and the signal relative to control plotted. Treatment with 10 nM or greater dexamethasone reduced TPH RNA levels by 5–6-fold (Student's *t*-test, two-tailed, paired, \* *P* < 0.05, 2 separate experiments). Equivalent results were obtained with 24 h treatment at 500 nM dexamethasone.

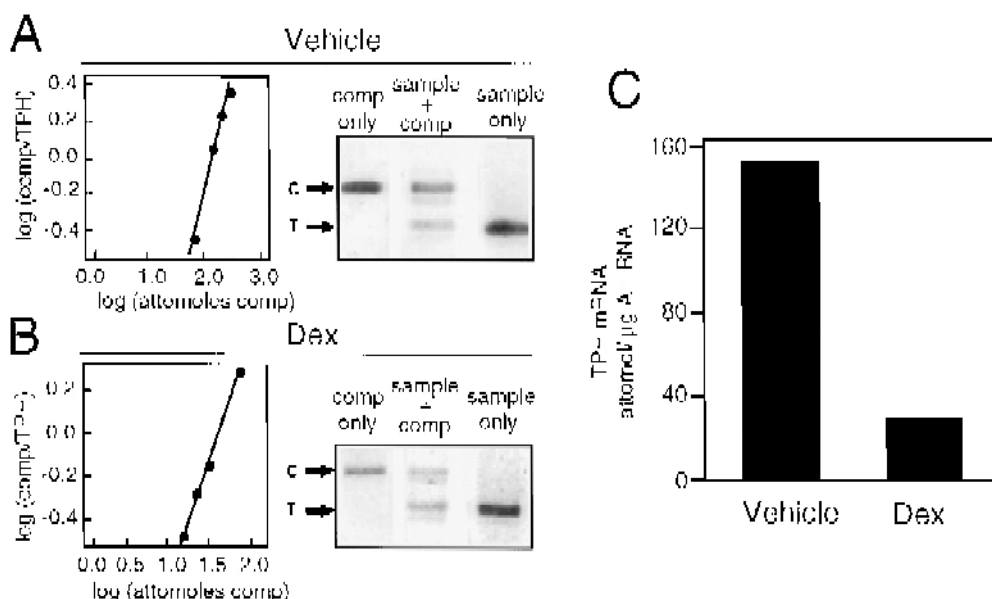


Fig. 5. Competitive RT-PCR produces results equivalent to those obtained by RNA slot-blots. The same RNA samples ( $1 \mu\text{g A}^+$ ) examined by slot-blot was analyzed by competitive RT-PCR. Competitor and TPH band densities were measured and analyzed as described in Fig. 2. Examples of the RT-PCR results from (A) control and (B) 10 nM dexamethasone-treated samples are shown, with lanes corresponding to competitor only, sample and competitor near equivalency, and sample only. A negative image of an ethidium bromide-stained gel is shown. C: RNA from control cells contained 153 amol TPH mRNA/ $\mu\text{g A}^+$  RNA, while that from cells treated with 10 nM dexamethasone contained 30.6 amol TPH mRNA/ $\mu\text{g A}^+$  RNA. Similar results were obtained from the 100 nM dexamethasone samples.

either 500 nM dexamethasone or vehicle for 5 days. The levels of both 5-HT and its major metabolite 5-HIAA (5-hydroxyindole acetic acid) were measured by HPLC. Vehicle-treated CA77 cells contained 80 pmol 5-HT and 85 pmol 5-HIAA per  $10^6$  cells (Fig. 6). The 5-HT level is similar to our previous measurements [9]. In contrast, both

5-HT and 5-HIAA levels in dexamethasone-treated cells were below the limit of detection,  $\approx 15 \text{ pmol per } 10^6$  cells. Thus, 5 days of dexamethasone treatment reduces 5-HT and 5-HIAA levels in CA77 cells by at least 5-fold.

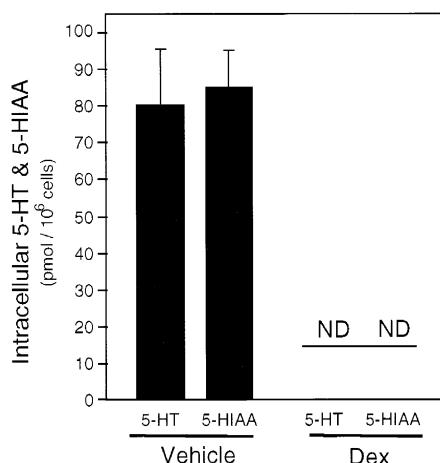


Fig. 6. Dexamethasone treatment decreases 5-HT and 5-HIAA levels in the CA77 cell line. CA77 cells were treated with either 500 nM dexamethasone or vehicle alone for 5 days, harvested, and assayed for 5-HT and 5-HIAA content. Both 5-HT and 5-HIAA were detected in vehicle-treated cells, but were below the limit of detection in the dexamethasone-treated cells. Limit of detection in this assay was  $\approx 15 \text{ pmol}/10^6$  cells (indicated by the horizontal bar). ND, not detectable. Error bars indicate S.E.M. from 5 vehicle- and 5 dexamethasone-treated (Dex) cultures (Student's *t*-test, two-tailed, paired,  $P = 0.018$ ).

#### 4. Discussion

In this study, we have tested the hypothesis that chronic glucocorticoid treatment is capable of regulating TPH mRNA levels. Using a sensitive RT-PCR assay, we have found that TPH mRNA levels are regulated by dexamethasone in a tissue-specific manner. After 1 week of dexamethasone treatment of adrenalectomized rats, we measured a robust increase of  $\approx 16$ -fold in the non-neuronal pineal gland that is contrasted by a significant decrease to  $< 20\%$  of the untreated levels in the neuronal raphe nuclei of the brain. While there are no other reports on chronic glucocorticoid regulation of TPH in the pineal or brain, there are data showing that acute glucocorticoid treatment and stress increase TPH protein and/or activity in the raphe [2,41,42]. In partial agreement with those reports, in our preliminary studies, we did not see a decrease in raphe TPH mRNA, yet did observe an increase in pineal TPH mRNA after 1 day of dexamethasone treatment. Hence these data demonstrate that there is a tissue-specific regulation of the TPH gene by dexamethasone, and furthermore, suggest that there is a delayed repression in the raphe.

What mechanisms could account for this tissue-specific delayed repression? One possibility is that dexamethasone

repression of TPH mRNA levels in the raphe is an indirect consequence of changes occurring outside the raphe. For example, a reduction in neurotrophin support could be occurring. Siuciak et al. [43] have reported that infusion of brain-derived neurotrophic factor (BDNF) into the dorsal raphe increases serotonergic activity and we have found that BDNF infusion increases TPH mRNA levels in the dorsal raphe (manuscript in preparation). Administration of corticosterone has been shown to decrease the expression of BDNF in the hippocampus [44]. Thus, it is possible that reductions in TPH mRNA levels in response to chronic dexamethasone treatment could reflect withdrawal of neurotrophin support. Alternatively, dexamethasone could also act directly since serotonin containing cells in the raphe nuclei have been reported to contain strong glucocorticoid receptor immunoreactivity [22], but other stimuli and/or a long message half-life would delay the decrease in TPH mRNA levels. The fairly rapid decrease in TPH mRNA levels in the homogenous serotonergic CA77 C cells argues for this second hypothesis. A third possibility is that the delayed response seen in vivo is due to both indirect and direct mechanisms. In either case, the CA77 C cell line provides a useful in vitro model system for mechanistic studies. In this respect, we have observed that transcription driven by 3.1 kb of TPH 5'-flanking DNA is repressed 2-fold by dexamethasone in CA77 cells, although this effect does not map to a discrete negative glucocorticoid responsive element (data not shown). In the pineal, similar direct and indirect mechanisms could also operate to stimulate TPH gene expression in response to dexamethasone. Further studies will be necessary to clarify these issues.

Regardless of mechanism, understanding regulation of TPH mRNA levels by chronic dexamethasone treatment may assist in understanding glucocorticoid effects upon sleep and mood. Clinically, exogenous glucocorticoids are capable of producing sleep and mood disturbances, including depression and anxiety [28,30,33,39,45]. The processes responsible for these effects are not known. Since both serotonin and melatonin are involved in regulation of sleep, it seems possible that alterations in their biosynthesis could alter sleep patterns. Interestingly, a significant number of depressed individuals also possess chronically elevated cortisol levels, not responsive to feedback controls [7,24,38]. However, some depressed individuals do not have elevated cortisol levels, and not all people given high dose glucocorticoids develop mood disorders [30]. Therefore, while it seems likely that multiple factors contribute to affective disorders, and depressive illness, reduced TPH mRNA levels have the potential to play a role in their pathogenesis.

## Acknowledgements

We thank Kit Bonson and Judith Siuciak for helpful and interesting discussions. We especially thank Judith Siuciak

for generously performing assays of 5-HT and 5-HIAA content in CA77 cells. This work was supported by NIH Grant HD25969, with tissue culture support from the Diabetes and Endocrinology Research Center (DK25295), and an individual NIMH NRSA (MH10752) to M.S.C.

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