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Daily Rhythm of Tryptophan Hydroxylase-2 Messenger Ribonucleic Acid within Raphe Neurons Is Induced by Corticoid Daily Surge and Modulated by Enhanced Locomotor Activity

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Tryptophan hydroxylase (TPH, the rate-limiting enzyme of serotonin synthesis) protein and mRNA levels display a circadian expression in the rat dorsal and median raphe. These patterns suggest a rhythmic synthesis of serotonin under the control of the master clock of suprachiasmatic nuclei. In the present study, we examined the involvement of endocrine and behavioral output signals of the master clock upon the *Tph2* mRNA levels by quantitative *in situ* hybridization. In the absence of adrenals, a complete suppression of *Tph2* mRNA rhythm was observed in dorsal and median raphe over 24 h. The restoration of corticosterone daily variations in adrenalectomized rats induced a *Tph2* mRNA rhythmic pattern *de novo*, indicating that *Tph2* mRNA rhythm is dependent upon daily fluctuations of glucocorticoids. Enhanced voluntary locomotor activity during 6 wk increased the level of *Tph2* mRNA in both raphe nuclei of control rats without concomitant increase of corticosterone plasma levels. Moreover, this long-term enhanced locomotor activity was able to restore significant variation of *Tph2* mRNA in adrenalectomized rats. In conclusion, both endocrine and behavioral cues can modulate *Tph2* expression in dorsal and median raphe. The corticosterone surge acts as a rhythmic cue that induces the rhythmic expression of *Tph2* in the raphe neurons. On the other hand, long-term exercise modulates the expression levels of this gene. Thus, the serotonin neurons are a target for both endocrine and behavioral circadian cues, and the serotoninergic input to the suprachiasmatic nuclei might feedback and influence the functioning of the clock itself. (*Endocrinology* 148: 5165–5172, 2007)

SEROTONIN (5-HT) IS involved in various physiological and behavioral functions (for review see Ref. 1). A number of these functions, including sleep, locomotor activity, and feeding behavior, have a time-related organization controlled by the central clock located within the suprachiasmatic nuclei (SCN). Moreover, the 5-HT input to the SCN, arising from raphe nuclei (2, 3), is known to modify the phase of the circadian pacemaker (for review see Ref. 4).

We have previously demonstrated daily variations of 5-HT release within the SCN of rats (5), associated with rhythmic protein and mRNA profiles of tryptophan hydroxylase (TPH), the rate-limiting enzyme in 5-HT synthesis (6) within median and dorsal raphe nuclei (MR and DR, respectively) (7, 8). The rhythm of *Tph2* mRNA levels, which has been shown to be circadian in nature (7), is thus under SCN control. The SCN are known to distribute circadian messages via neural, endocrine, and behavioral outputs (for review see Ref. 9). The question is thus to identify the SCN output signals responsible for the rhythmic *Tph2* gene expression.

The glucocorticoid daily surge is one of the most convincing candidates: 1) corticosterone circadian secretion is directly under SCN control (10), 2) glucocorticoid receptors are expressed in 5-HT neurons (11), and 3) several studies designed with stress paradigms or with glucocorticoid administration report an action on *Tph* mRNA, protein levels, and activity (12–18).

Locomotor activity, a behavior for which the temporal organization is also under SCN control, should also be considered especially because functional relationships have been established with the 5-HT system. For example, 5-HT neuronal firing rates and 5-HT release in several brain areas are highly correlated with behavioral states (19–21). The onset of locomotor activity also occurs at the beginning of the night period, when extracellular levels of 5-HT are at their highest in the SCN (5, 22). Moreover, access to a running wheel for several hours at midday acutely enhances 5-HT release, at least within the SCN (22). Finally, long-term voluntary access to a running wheel has been demonstrated to modulate the expression of several markers in 5-HT neurons (23).

This study was designed to determine whether hormonal and/or behavioral outputs of the SCN, namely the daily corticoid surge and locomotor activity, are involved in the daily rhythmic expression of *Tph2* mRNA within DR and MR. The *Tph2* gene expression was evaluated over 24 h after

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Abbreviations: ADX-Cort-10, Adrenalectomized rats given sc pellets containing 10% corticosterone and 90% cholesterol; ADX-Cort-R, ADX-Cort-rhythmic; DM, dorsomedian; DR, dorsal raphe nuclei; GR, glucocorticoid receptor; 5-HT, serotonin; LAT, lateral groups; MR, median raphe nuclei; NA, noradrenaline; SCN, suprachiasmatic nuclei; SSC, sodium saline citrate; TPH, tryptophan hydroxylase; VM, ventromedian; ZT, zeitgeber time.

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experimental manipulations of plasma corticosterone secretion. The effect of long-term voluntary exercise on *Tph2* mRNA expression was also assessed after access to a running wheel. Furthermore, because interactions between corticosterone release and long-term exercise have been previously described (24), the ability of enhanced locomotor activity to modulate the expression of *Tph2* was also investigated in the absence of the corticosterone daily surge.

Materials and Methods

Animal care

All experiments were performed in accordance with National Institutes of Health Guidelines regarding the care and use of animals for experimental procedures, with the European Communities Council Directive of November 24, 1986 (86/6.9/EEC), and French laws. Five-week-old male rats (Wistar; Charles River, L'Arbresle, France), weighing 180–200 g were housed under a 12-h light, 12-h dark cycle, with dim red light (<2 lux) continuously. Referential zeitgeber time zero (ZT0) was defined as the beginning of the light period. All animals were left for 1 wk in the animal facility before the experiments. During the experiment, animals were housed individually in transparent plastic cages and had free access to food and water (details below).

Surgery

Adrenalectomy was performed bilaterally according to a dorsal approach under deep anesthesia (Zoletil 20 mg/ml at 0.2 ml/100 g and Rompun 2% at 0.05 ml/100 g). After incising skin and muscles, adrenal glands were quickly removed, and sc pellets containing 10% corticosterone and 90% cholesterol (Sigma Chemical Chimie, Lyon, France) were implanted (ADX-Cort-10 rats). Sham animals were operated as described above but without removing adrenals and received 100% cholesterol control pellets. The pellets (12 mm length × 6 mm width × 4 mm diameter, weighing 240–260 mg) were prepared as described in a previous report (25). The 10% corticosterone pellets have been used in ADX-Cort-10 rats to provide a constant level of this hormone and at the corresponding diurnal amount measured in control rats (25, 26). ADX-Cort-10 rats were given drinking water containing 0.9% NaCl.

Animal treatments and brain collection

Corticosterone supplementation. To experimentally reinstate the nocturnal peak of corticosterone in ADX-Cort-10 rats, drinking water was replaced during the night (from ZT12 to ZT0), by a corticosterone-containing solution (50 μg/ml of corticosterone in 0.9% NaCl); this group of rats is named ADX-Cort-rhythmic (ADX-Cort-R). During daytime, the ADX-Cort-R rats had drinking water with 0.9% NaCl. These rats were supplemented in corticosterone during 1 wk and compared with sham and ADX-Cort-10 rats.

Sham, ADX-Cort-10, and ADX-Cort-R rats were killed 2 wk after surgery across the day/night cycle at the following time-points: ZT2, ZT6, ZT10, ZT14, ZT16, ZT18, and ZT23. Brains were quickly removed, frozen in cold isopentane, and stored at −80 C until section preparation. To verify whether adrenalectomy was effective, thymus glands were weighed. As expected, thymus hyperplasia (26, 27) was observed in ADX-Cort-10 rats (960 ± 20 mg), whereas corticosterone replacement in ADX-Cort-R rats reestablished the same range of thymus weight (650 ± 20 mg) as for the sham group (610 ± 20 mg).

Locomotor activity. Rats were housed individually in cages either with or without a running wheel (diameter = 30 cm). To ensure that the surgery has no effect upon locomotor activity, in addition to sham and ADX-Cort-10 rats, a control nonoperated group of rats was used for this study. During 6 wk, wheel-running locomotor activity was quantified using Dataquest III acquisition system (Mini-mitter, Sunriver, OR). Animals were killed at two time points, ZT2 and ZT10, at which the *Tph2* mRNA variation had already been characterized (7). Brains were quickly removed and frozen as described for the first experiment. As observed in the previous study, only ADX-Cort-10 rats showed hyperplasia of thymus glands that weighed 900 ± 40 and 920 ± 50 mg, respectively, for rats with and without access to a running wheel in their cages. All the sham and control rats exhibited the same range of thymus gland weights (with wheel: sham, 640 ± 50 mg, and control, 620 ± 40 mg; without wheel: sham, 660 ± 50 mg, and control, 540 ± 30 mg).

Blood sampling and corticosterone dosage

Because no stress effect on plasma corticosterone concentrations is expected in ADX-Cort-10 and ADX-Cort-R rats, trunk blood was collected at the moment of the decapitation. In sham and control animals, to avoid the effect of stress on corticosterone concentrations during the decapitation, *in vivo* blood sampling was performed by intracarotid cannulation using PE-50 polyethylene tubing (Instech Laboratories, Plymouth Meeting, PA). In addition, some ADX-Cort-10 rats used for the enhanced locomotor activity study were also cannulated in the carotid to ensure that this surgery had no effect on the running wheel behavior. For 3 d after cannulation, rats were handled gently twice daily to minimize handling stress on the day of sampling. Blood samples (200 μl) were collected on the fourth day at the same time points as euthanasia into heparin-containing tubes and centrifuged (2800 rpm at 4 C). The resulting serum was stored at −20 C. Circulating corticosterone concentrations were assessed in duplicates using a protocol adapted from the commercially available RIA kit (MP Biomedical, Loughborough, UK). The sensitivity limit of the assay was 7.7 ng/ml, and the reproducibility of the method was determined by evaluating intraassay variation (7.1%) and interassay variation (6.5%).

In situ hybridization procedure

Brains were cut in serial coronal sections (20 μm thick) with a cryostat (Leica Instruments GmbH, Nussloch, Germany) throughout the rostral part of the raphe, including DR and MR (interaural from +1.9 to +0.7 mm (28) and collected on sterile, gelatin-coated slides.

For *in situ* hybridization, sense and antisense riboprobes for Tph2 were obtained as described in Malek *et al.* (7). Probes were transcribed from the corresponding linearized plasmids using the appropriate polymerase (MAXI script; Ambion, Austin, TX) in the presence of [^{35}S]UTP (1250 Ci/mmol, Amersham Biosciences, Little Chalfont, UK). Hybridization was performed as described previously (7). Briefly, sections were postfixed in 4% formaldehyde for 10 min and acetylated twice for 10 min in 0.5% acetic anhydride in 0.1 M triethanolamine (pH 8.0). Thereafter, sections were rinsed, dehydrated, and air dried. Hybridization was carried out overnight at 54 C in humid boxes by depositing 100 μl riboprobes (300 pM) in a solution containing 50% deionized formamide, 2× sodium saline citrate (SSC), 1× Denhardt's solution, 0.25 mg/ml yeast tRNA, 1 mg/ml salmon sperm DNA, 10% dextran sulfate, and 10 mM dithiothreitol. After hybridization, the sections were treated with ribonuclease A for 30 min at 37 C (1.4 μg/ml; Sigma). After several stringency washes performed in 4× SSC, 2× SSC, 0.5× SSC, and 0.2× SSC, sections were dehydrated, air dried, and then exposed to an autoradiographic film (Kodak BioMax; Kodak, Rochester, NY). Some slides were dipped with nuclear emulsion (Hypercoat Emulsions; Amersham) diluted 1:2 in H_2O and exposed for 3 wk at 4 C. Emulsioned slides and films were developed using Kodak BioMax reagents.

Data analysis and statistics

Quantitative analysis of the autoradiograms was performed using a computerized analysis system (Biocom program RAG 200). Total OD was measured for each region of interest (MR and DR). The area of *Tph2* mRNA expression of each region was measured and was similar between all experimental groups. Nonspecific OD was measured for each section within mesencephalic areas where *Tph2* mRNA is not specifically expressed and then subtracted from total OD. By reference to radioactive microscales, the OD was converted to disintegrations per minute to evaluate the expression levels of *Tph2* mRNA within the regions of interest. Figure 1 illustrates *Tph2* mRNA detection after *in situ* hybridization followed by a nuclear emulsion at three representative regions of mesencephalic area containing raphe nuclei: caudal [*left panel*, interaural, +0.7 mm in Paxinos and Watson (28)], mid-rostrocaudal (*middle panel*, interaural, +1.2 mm) and rostral (*right panel*, interaural, +1.7 mm). DR is divided into three distinguishable subgroups in which *Tph2* mRNA levels were quantified separately: the ventromedian (VM) and

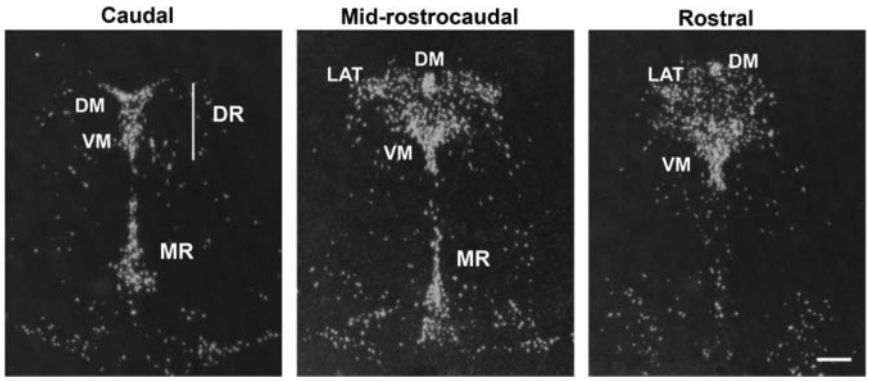

FIG. 1. *Tph2* mRNA detection by *in situ* hybridization and nuclear emulsion in DR and MR. Each *panel* is a representative caudal, mid-rostrocaudal, and rostral region of DR and MR. DR is anatomically divided into VM, DM, and LAT subdivisions. *Scale bar*, 280 μm.

the dorsomedian (DM) are present throughout the whole caudorostral extent of DR, whereas lateral groups (LAT) are in the mid-rostrocaudal and rostral parts of DR. For each brain and at each time point, *Tph2* mRNA was quantified in VM and DM over 10 sections at 100-μm intervals and in seven sections for LAT and MR.

One-way ANOVA was conducted to investigate the daily variations of *Tph2* mRNA levels in MR and DR subdivisions for sham, ADX-Cort-10, and ADX-Cort-R groups. *Post hoc* Newman-Keuls analysis was also performed to examine the significant differences between different time points. One-way ANOVA with repeated measures was performed to investigate the daily variation of plasma corticosterone concentrations in all the groups.

For the locomotor activity study, actograms were analyzed using ClockLab software (Actimetrics, Evanston, IL). Both daily and 6-wk analysis of locomotor activity were performed. Repeated-measures one-way ANOVA and paired Student's *t* test were used, respectively, to compare the daily and 6-wk distributions of locomotor activity between control, sham, and ADX-Cort-10 groups. Comparison of the mean values of *Tph2* mRNA levels as well as plasma corticosterone concentrations between ZT2 and ZT10 for ADX-Cort-10, sham, and control groups was assessed by using unpaired and paired Student's *t* test, respectively. For all statistical procedures, the level of significance was set at $P < 0.05$.

Results

Corticosterone rhythmic secretion and Tph2 mRNA daily profiles

Plasma corticosterone concentrations. In the sham rats, the daily profile of corticosterone concentrations exhibited the previously described pattern with increasing levels at the end of the light period (Fig. 2; one-way ANOVA, $P < 0.001$). Adrenalectomy completely suppressed the rhythmic pattern of corticosterone secretion. The constant blood concentration of this hormone measured in ADX-Cort-10 rats resulting from the 10% corticosterone pellets corresponded well to the diurnal level observed in sham rats (Fig. 2; one-way ANOVA, $P > 0.05$). In ADX-Cort-R rats, the 10% corticosterone pellet and nocturnal corticosterone intake of drinking water supplemented with 50 μg/ml corticosterone from ZT12 restored a daily pattern of plasma corticosterone concentration close to the sham group (Fig. 2; one-way ANOVA, $P < 0.001$). As the hormone oral intake in ADX-Cort-R rats began at ZT12, plasma corticosterone concentrations increased as from ZT14.

Effect of corticosterone on Tph2 mRNA daily profiles. For each experimental group, *Tph2* mRNA levels were quantified in MR and DR using *in situ* hybridization. In the sham group, *Tph2* mRNA presented a rhythmic profile with increased levels at ZT10 (Fig. 3; one-way ANOVA, $P < 0.01$ for MR and all DR subdivisions). Compared with ZT2, *Tph2* mRNA at ZT10 was increased by 36% in MR ($P < 0.001$), 41% in VM ($P < 0.001$), 44% in DM ($P < 0.01$), and 45% in LAT ($P < 0.001$). After adrenalectomy that led to the loss of corticosterone daily fluctuations, the rhythmic variation of *Tph2* mRNA in MR and in all DR subdivisions was completely abolished (Fig. 3; one-way ANOVA, $P > 0.05$). In contrast, in ADX-Cort-R rats for which daily plasma corticosterone concentration was reestablished, the rhythm of *Tph2* mRNA was restored (Fig. 3; one-way ANOVA, $P < 0.05$ for VM and LAT and $P < 0.01$ for MR and DM). The highest values of *Tph2* mRNA occurred at ZT16, and at that time point, compared with ZT2, the level was increased by 41% in MR ($P < 0.01$), 44% in VM ($P < 0.05$), 49% in DM ($P < 0.01$), and 46% in LAT ($P < 0.01$). It is noticeable that in sham rats, the increase of plasma corticosterone and *Tph2* mRNA levels was concomitant. Plasma corticosterone and *Tph2* mRNA increase was also time related in ADX-Cort-R rats but occurred later because corticosterone was given via a drinking solution after the onset of darkness.

Enhanced locomotor activity study

The effect of enhanced locomotor activity on *Tph2* gene expression was evaluated in sham, control, and ADX-Cort-10

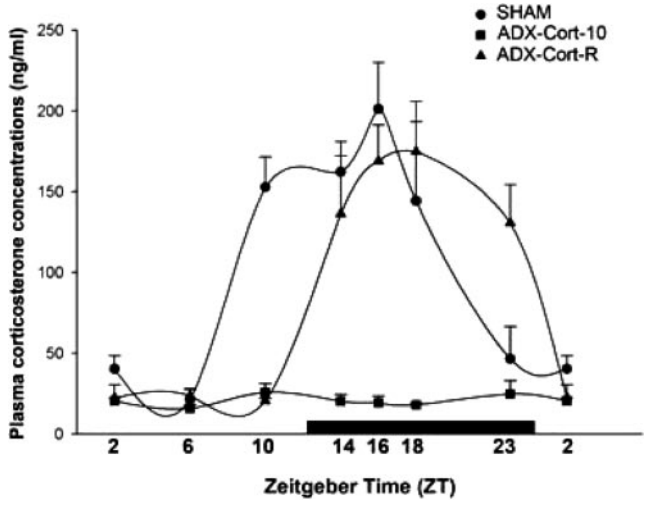

FIG. 2. Corticosterone plasma concentrations. Daily profiles of mean + SEM of corticosterone plasma levels (ng/ml) measured by RIA in sham (●; n = 5), ADX-Cort-10 (■; n = 6), and ADX-Cort-R (▲; n = 6) rats. The *black horizontal bar* represents the night period. By using one-way ANOVA, $P < 0.001$ for the time-point effect on corticosterone plasma concentrations in SHAM and ADX-Cort-R rats.

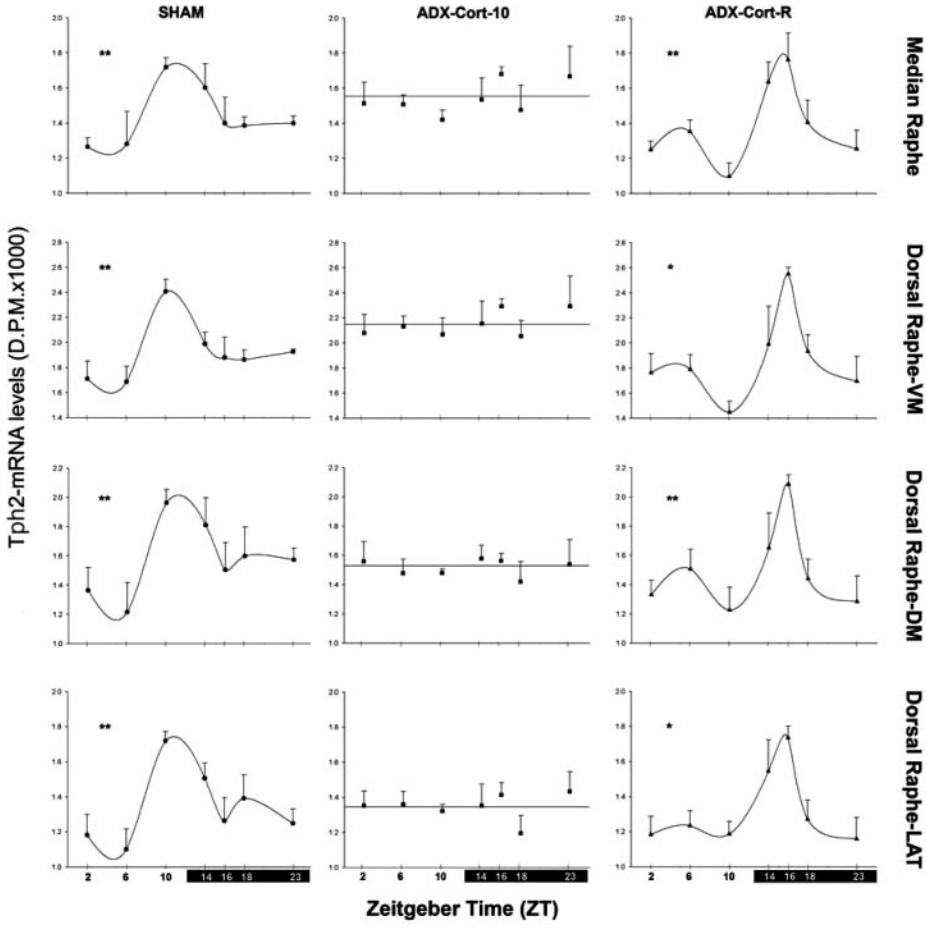

FIG. 3. Rhythmic expression of *Tph2* mRNA: effect of adrenalectomy and hormonal replacement. Shown are daily profiles of *Tph2* mRNA levels in MR and DR subdivisions, VM, DM, and LAT, after quantitative *in situ* hybridization in sham, ADX-Cort-10, and ADX-Cort-R rats. Each *symbol* is the mean + SEM of four to six rats in which *Tph2* mRNA was quantified throughout the whole caudorostral extent of each subdivision. The *black horizontal bar* represents the night period. *, $P < 0.05$; **, $P < 0.01$ using one-way ANOVA to assess the effect of the time point on *Tph2* mRNA expression variation.

rats housed during 6 wk in individual cages with or without a running wheel. As expected, sham animals housed in cages without a wheel showed the previously described (see above and Ref. 7) significant increase of *Tph2* mRNA levels between ZT2 and ZT10 (SHAM in Fig. 4; *t* test, $P < 0.01$ in MR and all DR subdivisions). The percentage of this increase was 30% in MR, 35% in VM, 32% in DM, and 31% in LAT. The differences in the absolute values between Figs. 3 and 4 are due to the fact that they were collected in two separate experiments. The percentages of variation, however, are in line with previous findings (7) and within the same range as those in the first experiment. The enhanced locomotor activity in sham rats (with wheel) induced over a 2-fold increase in the amplitude of ZT2/ZT10 variation of *Tph2* mRNA: 70% in MR, 79% in both VM and DM, and 74% in LAT (Fig. 4; *t* test, $P < 0.001$ for MR and all DR subdivisions). The increased amplitude of ZT2/ZT10 variation was mainly due to the up-regulation of *Tph2* mRNA expression at ZT10 in sham animals with a running wheel (*t* test, ZT10 with *vs.* ZT10 without a wheel: $P < 0.05$ for MR, $P < 0.01$ for VM, and $P < 0.001$ for DM and LAT). Indeed, no significant difference was observed in *Tph2* mRNA expression in sham rats at ZT2 with and without a wheel (*t* test, $P > 0.05$). The effect of the running wheel on *Tph2* mRNA expression in control rats was similar, at both time points, to that reported for sham group (data not shown).

To investigate whether the effect of voluntary exercise on *Tph2* mRNA expression was mediated by an increase of corticosterone secretion we measured plasma corticosterone concentrations. Sham and control rats display the same range of corticosterone concentrations regardless of the presence or the absence of a wheel in the cage at both ZT2 and ZT10 time points (Table 1).

In line with our first experiment, adrenalectomy abolished the ZT2/ZT10 variation of *Tph2* mRNA in rats housed 6 wk without a running wheel (Fig. 4; *t* test, $P > 0.05$ for MR and DR). ADX-Cort-10 rats with access to a running wheel displayed *de novo* a significant variation of *Tph2* mRNA between ZT2 and ZT10 (Fig. 4; *t* test, $P < 0.001$ for MR, VM, and LAT, $P < 0.05$ for DM). The percentage of this newly induced variation between ZT2 and ZT10 was 40% in MR, 25% in VM, and 34% in both DM and LAT. Statistical analysis demonStrated that this variation was due to the lower level of *Tph2* mRNA expression at ZT2 compared with animals with no access to a wheel (*t* test, ZT2 with *vs.* ZT2 without wheel: $P < 0.001$ for MR, DM, and LAT, $P < 0.01$ for VM). Comparing *Tph2* mRNA expression in ADX-Cort-10 rats at ZT10 with and without a wheel showed no significant difference (*t* test, $P > 0.05$).

Comparative quantification of wheel-running activity was conducted in ADX-Cort-10, sham, and control rats. Intracarotid cannulation had no effect on the rhythm of running wheel activity (data not shown). The clear day/night organization of wheel locomotor activity was similar in all ex-

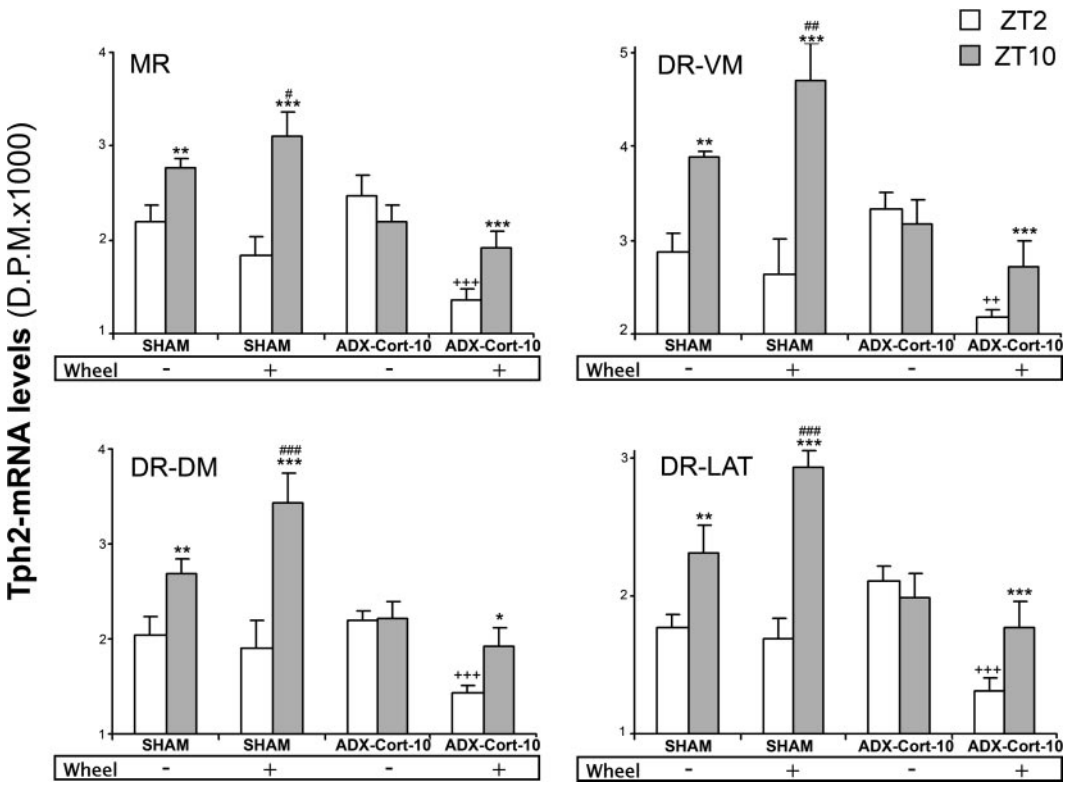

FIG. 4. Expression of *Tph2* mRNA after 6 wk of voluntary exercise. *Tph2* mRNA levels were quantified after *in situ* hybridization in sham and ADX-Cort-10 rats with and without a running wheel at ZT2 (*white bars*) and ZT10 (*gray bars*). *Tph2* mRNA levels were measured in MR and DR subdivisions VM, DM, and LAT. Each *vertical bar* corresponds to the mean + SEM of four to six rats. The *symbols* *, #, and + illustrate, respectively, the difference between ZT2/ZT10 in all groups, ZT10/ZT10 in sham groups, and ZT2/ZT2 in ADX-Cort-10 groups. The *t* test significance was set at $P < 0.05$ (*one symbol*), $P < 0.01$ (*two symbols*), and $P < 0.001$ (*three symbols*).

perimental groups (Fig. 5, A and B). However, when considering the 6-wk duration of the experiment, adrenalectomy induced a significant decrease of wheel-running activity, which is the result of a reduction of the nocturnal activity in ADX-Cort-10 rats (*t* test, $P < 0.01$). A week-by-week analysis of the wheel-running activity (Fig. 6) highlights the higher level in sham rats compared with ADX-Cort-10 during the first 4 wk (one-way ANOVA, $P < 0.001$ for wk 1, 2, 3, and 4). By the end of wk 6, both groups showed the same level of wheel-running activity (Fig. 6; one-way ANOVA, $P > 0.05$ for wk 5 and 6). Rats in the control group exhibited the same pattern of locomotor activity described for sham rats (data not shown).

Discussion

In the present study, we evaluated the capacity of two SCN-controlled rhythmic cues, corticosterone and enhanced locomotor activity, to regulate the daily variation of *Tph2* mRNA in raphe nuclei. We demonstrate for the first time the involvement of these hormonal and behavioral cues in the rhythmic expression of *Tph2* mRNA in DR and MR. Furthermore, our experimental paradigm enables us to dissociate the effects of both parameters, which modulate *Tph2* mRNA expression in a different way.

Corticosterone effect on Tph2 expression

The present study demonstrates that the corticosterone daily pattern is responsible for the rhythm of *Tph2* mRNA in the rat DR and MR. *Tph2* mRNA exhibited a daily rhythmic expression in all DR subdivisions and MR, as previously described (7). After adrenalectomy, *Tph2* mRNA is expressed at a constant level through 24 h in DR and MR, suggesting that the corticosterone surge might drive the rhythmic pattern of *Tph2* mRNA. This issue is further confirmed by the fact that the *Tph2* mRNA daily pattern is fully restored in ADX-Cort-10 rats after addition of corticosterone in the drinking water during night (a manipulation that reinstates the daily rhythm of corticosterone). Furthermore, our results clearly demonstrate that plasma corticosterone and *Tph2* mRNA rhythms are time related because a concomitant in-

TABLE 1. Plasma corticosterone concentrations (ng/ml) at ZT2 and ZT10 after blood sampling by intracarotid cannulation performed in sham-operated and control rats housed with or without a running wheel (n = number of rats at each time point)

	ZT2		ZT10	
	Without wheel	With wheel	Without wheel	With wheel
Sham	33 ± 8 (n=4)	33 ± 4 (n=6)	148 ± 19 (n=4)	130 ± 16 (n=7)
Control	23 ± 2 (n=4)	36 ± 11 (n=4)	129 ± 20 (n=4)	137 ± 28 (n=5)

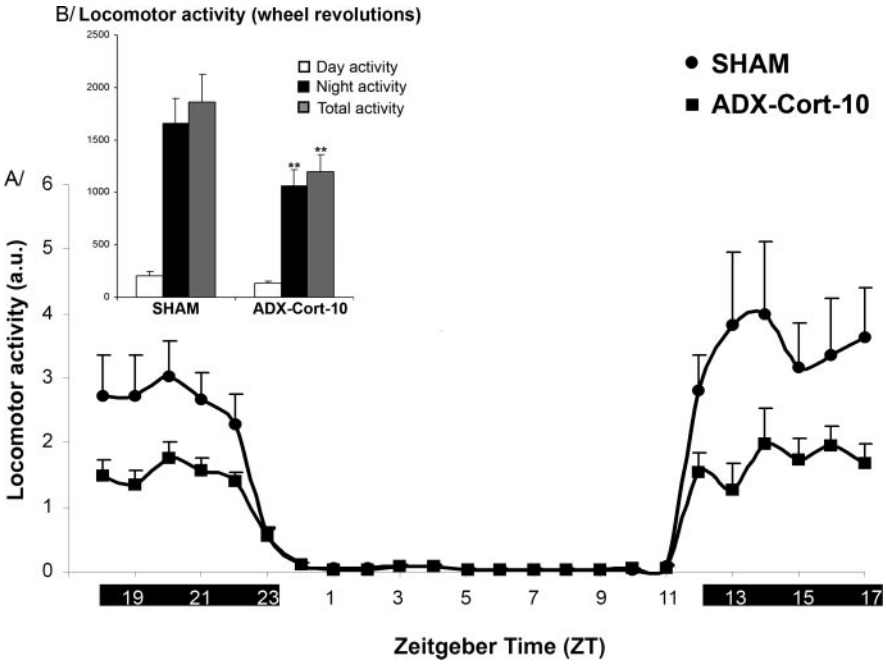

FIG. 5. Wheel-running activity of sham and ADX-Cort-10 rats. A, Daily organization of running activity during 6 wk in sham (●, n = 11) and ADX-Cort-10 (■, n = 12) rats. Each *symbol* is the mean value over 6 wk, and the *black bar* represents the night period. B, The mean locomotor activity during day (*white*), night (*black*), and total (*gray*, *i.e.* sum of day plus night activity) measured as the number of wheel revolutions in the sham and ADX-Cort-10 rats. **, $P < 0.01$ for the effect of adrenalectomy on night and total locomotor activity, by *t* test.

crease of both parameters is observed in sham as well as in ADX-Cort-R rats. Altogether, these results clearly establish the role of the daily corticosterone fluctuations in the rhythmic pattern of *Tph2* mRNA in the raphe.

Earlier studies have shown that 5-HT neurons are sensitive to glucocorticoid and that this hormone modulates intrinsic factors of 5-HT synthesis (12–16, 18). However, regarding *Tph* mRNA expression, reports are conflicting with either increase, decrease, or no change after glucocorticoid treatment, immobilization, or social stress (13–15, 29). The fact that in these earlier studies the rhythmic pattern of both *Tph* mRNA expression and endogenous glucocorticoids were not considered can explain to some extent these discrepancies. It should also not be forgotten that until recently, only the nonneuronal *Tph1* gene expression was considered, *Tph2* being poorly documented. The present study is the first, to our knowledge, that investigates through the 24-h cycle the effect of endogenous corticoid fluctuation on *Tph2* mRNA.

The mechanisms involved in corticosterone induction of *Tph2* mRNA circadian expression are not known. An indirect action of corticoids, for example via glucocorticoid-sensitive brain areas projecting to the raphe nuclei, cannot be excluded. For example, noradrenaline (NA) neurons of the locus coeruleus are sensitive to corticoids (30) and are responsible for a tonic NA input to the 5-HT neurons (31, 32). NA through actions on NA receptors modulates the firing rate of 5-HT neurons as well as 5-HT synthesis and release (31, 33, 34). A direct action of corticosterone on 5-HT neurons remains, however, the most expected hypothesis. Corticosterone acts through glucocorticoid receptors (GRs) or mineralocorticoid receptors. The involvement of mineralocorticoid receptors in this response is unlikely because these receptors are not present in raphe nuclei (35). GRs, however, have been demonstrated to be coexpressed with 5-HT and TPH in raphe neurons (11, 36). Generally speaking, it is known that to act upon gene expression, corticoids are sub-

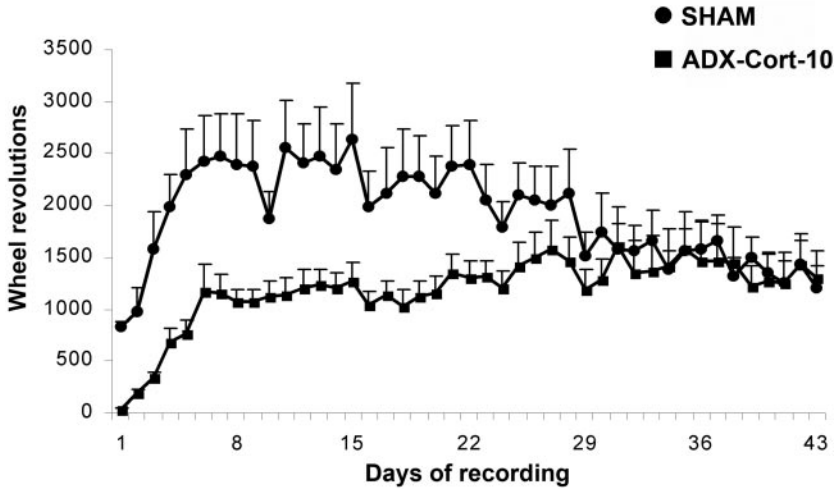

FIG. 6. Week-by-week analysis of the wheel-running activity of sham and ADX-Cort-10 rats. Amount of wheel-running activity in the same animals as described just above across the 6-wk period of the experiment. Using one-way ANOVA, $P < 0.001$ for the difference between sham and ADX-Cort-10 animals during the first 4 wk.

ordinated to a dynamic regulation of both GR and its principal intracellular chaperone, heat-shock protein 90. To determine whether the same mechanism takes place in the control of the rhythmic expression of *Tph2* gene, it would be necessary to investigate whether the expression of GR-heat-shock protein 90 complex is also rhythmic in the 5-HT raphe neurons. This is likely because it has already been described in hippocampus (37) but remains to be demonstrated in raphe neurons. The effect of corticoids on *Tph2* gene expression might also affect posttranscriptional processing and mRNA stability as previously described for other genes (for review see Ref. 38).

In the context of circadian functions, previous reports have demonstrated that corticosterone daily rhythm acts as a temporal signal able to sustain the expression of numerous rhythmic genes (39–42). Our present data also demonstrate that corticoids induce the daily *Tph2* mRNA expression. Although the present work describes the action of corticoids only at the mRNA level, our previous studies have established a temporal relationship between *Tph2* mRNA, protein, and neurotransmitter release, at least in the MR-SCN pathway (5, 7, 8). Thus, glucocorticoids could indirectly mediate a temporal signal throughout the brain via the widespread 5-HT innervation. The present findings can also explain the modulatory effect of glucocorticoids on the photic synchronization of locomotor activity reported by Sage *et al.* (26). Such an effect, which cannot result from a direct effect of glucocorticoids on the SCN cells because GRs are very low or absent (43), can be explained by an indirect effect of glucocorticoids via the 5-HT neurons. Indeed, the authors have reported the involvement of SCN 5-HT afferent fibers in this mechanism. Taken together, our data suggest that corticosterone circadian signal, through a direct or indirect effect on 5-HT raphe neurons, can affect physiological, behavioral, or emotional functions that are dependent on brain structures receiving 5-HT fibers.

Effect of enhanced locomotor activity on tph2 expression

Six weeks with access to a running wheel induces roughly a 2-fold increase in *Tph2* mRNA levels in DR and MR at ZT10 in sham and control rats. The same voluntary exercise has previously been shown to influence the serotoninergic transmission. After 6 wk with a running wheel, for example, modifications of the 5-HT transporter and 5-HT1A receptor mRNA levels have been reported within the DR and MR (23). The same group (44), however, described that *Tph2* mRNA expression was unaffected by enhanced locomotor activity, an observation that apparently contradicts our results. This is only an apparent contradiction. Indeed, Foley *et al.* (44) used rats killed during the morning, corresponding to our experimental time point ZT2 (1000 h) at which we report no differences in *Tph2* gene expression either. It is the analysis at different periods of the light/dark cycle that allowed us to detect this difference between the animals with and without a running wheel.

We had initially postulated that the 2-fold increase in *Tph2* mRNA might be the consequence of an increase of plasma corticoids induced by enhanced locomotor activity. However, at the end of the 6 wk, plasma corticosterone concentrations were similar in rats with and without a running wheel, which is in agreement with a previous study demonstrating that after 4 wk of voluntary exercise, a normal daily range of corticosterone level is already restored (24). Therefore, the up-regulation of *Tph2* mRNA expression observed at ZT10 after 6 wk of access to a running wheel might not be related to an increase of corticoid levels. Because long-term enhanced activity might induce modifications of GR expression and/or sensitivity, a role of this hormone cannot be totally excluded. Some previous data already suggested that *tph* gene expression can be modified by a glucocorticoid-independent mechanism (13, 15). Our present study also demonstrates that enhanced locomotor activity is effective in modifying the level of *Tph2* mRNA expression even in adrenalectomized rats, and interestingly, the observed ZT2/ZT10 variation of *Tph2* mRNA expression in ADX-Cort-10 rats is the consequence of a decrease at ZT2. It might be suggested that the different effect observed at ZT2 between sham and ADX-Cort-10 rats is the consequence of a phase shift of the rhythmic expression of *Tph2* mRNA in ADX-Cort-10 rats with a running wheel. This is unlikely because at the behavioral level, adrenalectomy had no influence on the rhythmic organization of the daily locomotor activity. The locomotor activity effect on *Tph2* mRNA levels appears to be complex and is triggered by a mechanism that is corticoid independent.

In conclusion, our results demonstrate the involvement of two different rhythmic outputs of the SCN, namely corticosterone daily surge and locomotor activity, in the regulation of the circadian profile of *tph2* gene expression in DR and MR. Corticosterone daily fluctuations control the rhythmic expression of *Tph2* mRNA levels, and the enhanced locomotor activity acts to modulate the level of *Tph2* mRNA in the raphe. The serotoninergic input to the SCN might be considered as an intermediate target for both endocrine and behavioral feedback on the clock itself. This effect might be considered in a more general context in which the common 5-HT projection areas in the brain would also provide a time-related signal over a 24-h cycle.

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