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Circadian rhythm of type II 5'deiodinase activity in the rat hypothalamic-pituitary-adrenal axis

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Type II 5' deiodinase (5'D-II) activity was assessed in the hypothalamus (HP), pituitary (PIT), and adrenal gland (AG) of adult male rats after two different photoperiodic regimes: (1) cyclic light-dark conditions (LD, 12:12) or (2) 4 days in continuous darkness (DD). Under cyclic LD conditions HP, PIT and AG 5'D-11 activity showed a significant circadian rhythm ($P<0.004-<0.0001$). The acrophases obtained by cosinor analysis were: HP, 12:37; PIT, 03:04 and AG, 02:30 h. In contrast, after 4 days in DD, HP 5'D-II rhythmicity was abolished and there was a significant shift in the acrophase of both PIT and AG enzyme activity. These results suggest that the 5'D-II oscillation in PIT and AG corresponds to an endogenous circadian rhythm and is not only a response to the presence of a L/D cycle.

Keywords: 5'deiodinase type II; circadian rhythm; hypothalamus; pituitary; adrenal gland; corticosterone

Introduction

Enzymatic variations in rhythmicity mainly in the circadian range have been described in different murine tissues (Feuers *et al.*, 1984, 1986, 1987; Marques *et al.*, 1994) and organelles (Harisch *et al.*, 1980). The circadian rhythmicity exhibited by type II 5' deiodinase (5'D-II) activity in several neuroendocrine organs seems to represent a conspicuous functional distinction among the two isoenzymes that catalyze outer-ring thyronine deiodination. With the exception of brown adipose tissue (Murakami *et al.*, 1988a) and the controversial reports regarding the anterior pituitary gland (Murakami *et al.*, 1988b; Guerrero *et al.*, 1988), rat 5'D-II circadian rhythmicity has been documented in the cerebral cortex (Guerrero *et al.*, 1988), as well as in the pineal and the Harderian gland (Guerrero *et al.*, 1988, 1992; Greer *et al.*, 1991). Furthermore, although its precise physiologic role is not yet elucidated, it has been well established that pineal 5'D-II activity is photoneural-dependent and primarily driven by β-adrenergic mechanisms (Greer *et al.*, 1991; Guerrero *et al.*, 1992).

Previous studies from our laboratory (Anguiano *et al.*, 1991) have shown that acute cold-exposure in the rat elicits the immediate (≈15 min) increase of hypothalamic and adrenal total 5'deiodinase activity (5'D). We previously reported that the major isoenzyme in the adrenal gland corresponds to 5'D-II (Luna *et al.*, 1993, 1995 in press) and presents a circadian variation (Luna *et al.*, 1992). The present study was designed to examine whether 5'D-II activity exhibits a truly circadian rhythm in those neuroendocrine structures that conform the so-called hypothalamic-pituitary-adrenal system (HPA). The specific aims were: (a) to analyse the 24 h profile of 5'D-II activity under cyclic lighting conditions, and (b) to compare the effect that exposure to continuous darkness (free-running conditions) exerts on this profile.

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Results

Cyclic lighting conditions

On the day of the experiment, groups of rats (five animals each) maintained under cyclic lighting conditions (12:12 LD) were decapitated at intervals of 3 h beginning at 06.00 h. As depicted in Figure 1, 5'D-II activity in all the analysed tissues presented a conspicuous significant variation with time of day ($P<0.004-<0.0001$; one-way ANOVA). In the HP the lowest significant values (6.4 ± 1.2 fmol/mg protein/h) were detected at 03:00 h ($P<0.004$), thereafter activity steadily increased, reaching its highest values around 12:00 h (20.7 ± 5.8 fmol/mg protein/h). The computed acrophase (cosinor analysis) was very close to the observed value, namely 12:37 h, with low amplitude (Table 1). Within the light period (6:00 to 18:00 h) there were minor differences in PIT and AG 5'D-II activity. However, after lights were off, enzyme activity steadily rose in both organs reaching maximal values at 03:00 h (2.8 ± 0.2 pmol/mg protein/h and 384 ± 43 fmol/mg protein/h in PIT and AG, respectively). The computed acrophases were 03:04 and 02:30 respectively (Table 1). Concomitantly, the corticosterone circulating level exhibited its well-known circadian rhythm which reached maximum values at 18:00 h (367.7 ± 28 ng/ml). The computed acrophase was 21:21 h (Table 1).

Continuous darkness

After 4 days in continuous darkness, animals (five per group) were decapitated at the same time intervals as in the LD group. This photoperiodic regime completely disrupted the nyctohemeral profile of HP 5'D-II activity, and was accompanied by a significant shift in the acrophase of both, PIT and AG enzyme activity and corticosterone rhythms (Figure 2 and Table 1). When photoperiod and time of day were used as variables (two-way ANOVA), mean values of 5'D-II activity tended to increase in PIT (1.3 ± 0.70 to 2.0 ± 0.06 pmol/mg protein/h), and were significantly higher in AG (188.6 ± 18 to 264.5 ± 9.2 fmol/mg protein/h), while circulating corticosterone levels were reduced (243.5 ± 12 to 190.1 ± 11 ng/ml) ($P<0.001$).

Discussion

Present results disclosed two major findings: (1) HP, PIT and AG 5'D-II activity under cyclic L/D conditions show marked diurnal variations, which, in the case of HP, are characterized by an advanced phase-shift (≈14 h) and (2) continuous darkness abolishes HP rhythmicity completely, and significantly shifts the acrophase of enzyme activity in PIT and AG. These findings strongly suggest that the 5'D-II oscillation in PIT and AG correspond to endogenous circadian phenomena and are not only a response to the presence of an L/D cycle.

As far as we know, this is the first study that analyses 5'D-II rhythmic activity in the hypothalamus. Nevertheless, our finding that hypothalamic deiodinase activity reaches its highest values during the light phase coincides with the well-

Table 1 Summary of circadian rhythm characteristics of 5'D-II activity and corticosterone*

	One way analysis of variance				*Single Cosinor analysis*				
Organ	*Light regime*	*Mean ± SEM*	*F ratio*	*P value*	*PR %*	*P value*	*Mesor ± SEM*	*Amplitude ± SEM*	*Acrophase (Φ in clock hours)*
HP	L/D	14.9±1.0	4.4	<0.003	17.8	<0.043	13.8±1.2	4.6±1.7	12.37
HP	D/D	15.7±1.0	1.5	NS	6.8	NS	15.9±1.0	2.3±1.5	–
PIT	L/D	1.3±0.70	5.0	<0.001	36.2	<0.001	1.8±0.8	0.5±0.1	03:04
PIT	D/D	2.0±0.06	28.0	<0.0001	34.7	<0.049	2.0±0.19	0.7±0.3	16:06
AG	L/D	188.6±92	17.0	<0.0001	57.6	<0.001	188.6±12	121.9±18	02:30
AG	D/D	264.5±23	5.2	<0.0007	45.8	<0.001	292.1±32	239.9±43	12:10
Corticosterone levels	L/D	243.5±12.2	4.0	<0.003	27.2	<0.002	244.5±13.0	73.5±18.4	21:21
Corticosterone levels	D/D	190.1±11.3	6.7	<0.005	53.1	<0.001	195.5±12.1	98.3±16.8	19:08

*Under two different photoperiodic regimes: L/D, 12:00 light/12:00 dark; D/D continuous darkness. HP, hypothalamus; PIT, pituitary; AG, adrenal gland; corticosterone N.S. not significant. Mean and MESOR are expressed in fmol I released/mg protein/h for HP and AG, and in pmol/mg protein/h for PIT; in the case of corticosterone these parameters are expressed in ng/ml. Φ = referes to light onset (6:00 A.M.). PR = percent rhythm.

known diurnal patterns exhibited by the TRH contents in HP as well as by the TSH, T3 and T4 levels of the so-called hypothalamic-pituitary-thyroid axis (Kerdelhue *et al.*, 1981; Ottenweller & Hedge, 1982; Martino *et al.*, 1985). In this context, previous reports showed that the time of zenith for TRH is at 14:00 h and around noon for TSH, which is followed by peaks of plasma T3 and T4 levels approximately 3–4 h later (Rookh *et al.*, 1979; Jordan *et al.*, 1980). Furthermore, our data showing that 4 days of constant darkness markedly dampen this HP rhythmicity, agree with previous reports in which constant darkness abolishes the diurnal variation in total hypothalamic TRH content as well as decreases the rhythmicity of TSH circulating levels (Kerdelhue *et al.*, 1981; Martino *et al.*, 1985). This sensitivity of HP 5'D-II activity to continuous darkness is in contrast with present results in PIT and AG enzyme activity, as well as with previous studies in which, exposure to short-term additional continuous darkness does not affect the nyctohemeral enzyme rhythmicity neither in pituitary nor in pineal gland (Murakami *et al.*, 1988b), whereas extension of light into the nocturnal phase of the cycle, completely inhibits the pineal nocturnal peak and significantly enhances enzyme rhythmicity in brain frontal cortex (Guerrero *et al.*, 1988, 1992). Altogether, these findings allow the suggestion that HP 5'D-II rhythmic activity is tightly linked to environmental light.

Present findings regarding 5'D-II rhythmicity in the PIT gland, confirm the results by Murakami *et al.* (1988a), and suggest that the disagreement with those studies in which this nyctohemeral rhythm was not found (Guerrero *et al.*, 1988), may be explained by differences in the photoperiodic schedule. Furthermore, present results demonstrate that under cyclic 12:12 light-dark conditions the acrophase of 5'D-II activity in PIT and AG (03:04 and 02:30 h respectively) is practically identical, and is in opposite phase (≈12 h) with the well known diurnal pattern exhibited by TSH, T4 and T3 (acrophases: 11:30, 12:56 and 13:46 h respectively) (Rookh *et al.*, 1979; Jordan *et al.*, 1980; Kerdelhue *et al.*, 1981; Martino *et al.*, 1985) as well as with the corticosterone levels that precede the acrophase of enzyme gland activity by ≈6 h. These results suggest that the PIT enzyme rhythm may be regulated in part by the inverse relationship between 5'D-II activity and substrate supply (Kaplan, 1984); as well as by the inhibitory effect that glucocorticoids exert on TSH secretion and extrathyroidal T3 production (Scanlon, 1991). In support of this interpretation, studies in hypothyroid animals have shown that the nyctohemeral rhythm of TSH and 5'D-II activity in PIT either disappears or is concealed (Fukuda *et al.*, 1975; Tonooka & Kobayashi, 1980; Murakami *et al.*, 1988a). Altogether, these data are consistent with the notion that PIT 5'D-II activity modulates the thyrostat set point (Greer *et al.*, 1991).

On the other hand, present results show both, that after 4 days under constant darkness, 5'D-II activity in PIT and AG is increased and that the enzyme acrophase in both glands is significantly dissociated (4 h). These results contrast with the decrease in corticosterone levels which nyctohemeral profile remains virtually constant (Fischman *et al.*, 1988; Ixart *et al.*, 1977). These findings are in agreement with an endogenous rhythm under a free-running condition, and strongly suggest that lights-on may be a Zeitgeber for 5'D-II activity in both structures. The phase shift and the dissociation of the enzyme activity in PIT and AG, can be explained by previous evidence in which the rhythmicity in TRH-TSH-TH levels (HP-PIT-thyroid axis) are decreased by continuous darkness while the HP-PIT-AG axis hormones remain constant. This adds further support to the participation of thyroid hormone supply as the main regulatory influence of 5'D-II nyctohemeral rhythm in these organs and suggests their role as an entertaining signal for the enzyme. However the persistence of circadian 5'D-II rhythmicity in PIT and AG, strongly suggests that it does not depend exclusively upon thyroid hormone supply. In this context, previous studies (Anguiano *et al.*, 1991; Valverde-R *et al.*, 1991) and a recent results (Anguiano *et al.*, 1995, in press) in which adrenal gland denervation results in a significant ipsilateral reduction in AG 5'D-II activity, suggest that, as in the case of the pineal and Harderian gland (Guerrero *et al.*, 1987, 1988, 1992; Murakami *et al.*, 1988b), AG enzyme activity rhythmicity could be primarily regulated by its tonic sympathetic input. Nonetheless, the presently compiled data only allow the proposal of these regulatory paths for rhythmicity of the HP-PIT-AG axis and studies are under way to determine the mechanism(s) which control this process.

In summary, present results demonstrate that under cyclic LD conditions HP, PIT and AG 5'D-II activity exhibits a significant circadian rhythm, which, in the case of PIT and AG, seems to correspond to an endogenous oscillation entrained by light.

Materials and methods

Reagents

Nonradioactive iodothyronines were obtained from Henning Co (Berlin, Germany) and corticosterone from Sigma Chemical Co. (St Louis, MO). ^{125}I-labeled thyroxine (T4) and reverse triiodothyronine (rT3), as well as 1,2,6,7 -^{3}H-hydrocortisone (sp. a. 1200, 1174 and 220 μci/ug, respectively) were purchased from New England Nuclear (Boston. MA). Dithiothreitol (DTT) was obtained from Calbiochem (La Joya, CA), and 6-n-propyl-2-thiouracil (PTU) from US. Biochemical Corp. (Cleveland, OH).

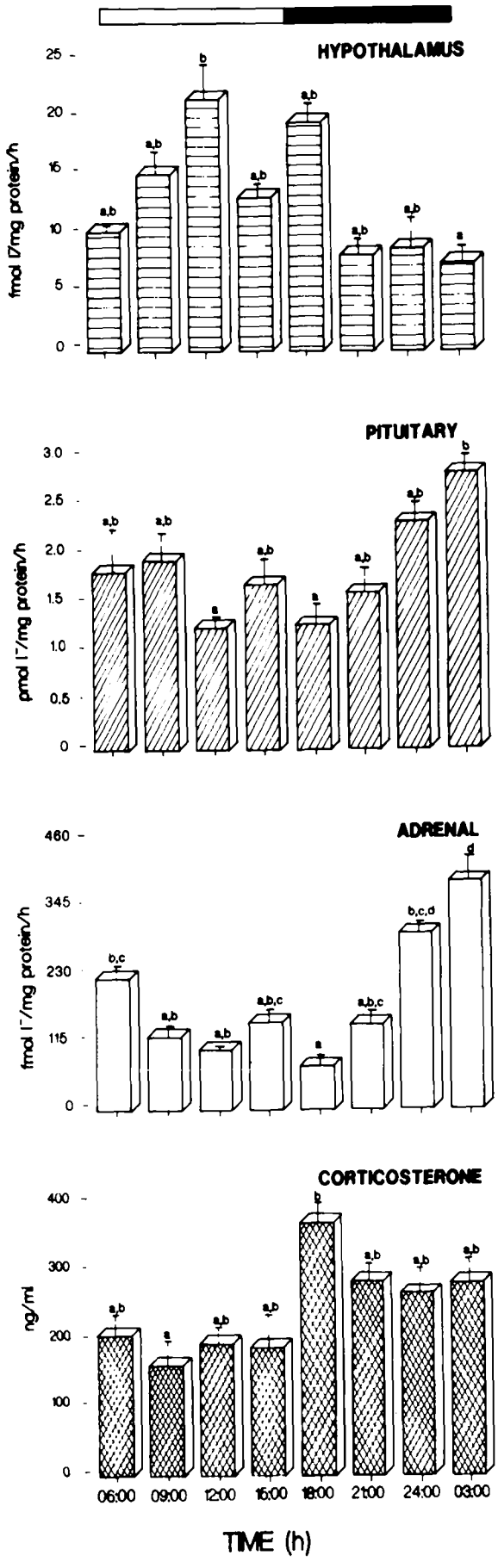

Figure 1 Circadian rhythm of HP, PIT and AG 5'D-II activity and circulating corticosterone levels in rats maintained under cyclic (12:12) light:dark conditions. Each bar represents the mean ± S.E. of enzyme activity quantified in five rats per group from three separate experiment. Means bearing different superscript letters are significantly different. (one-way ANOVA)

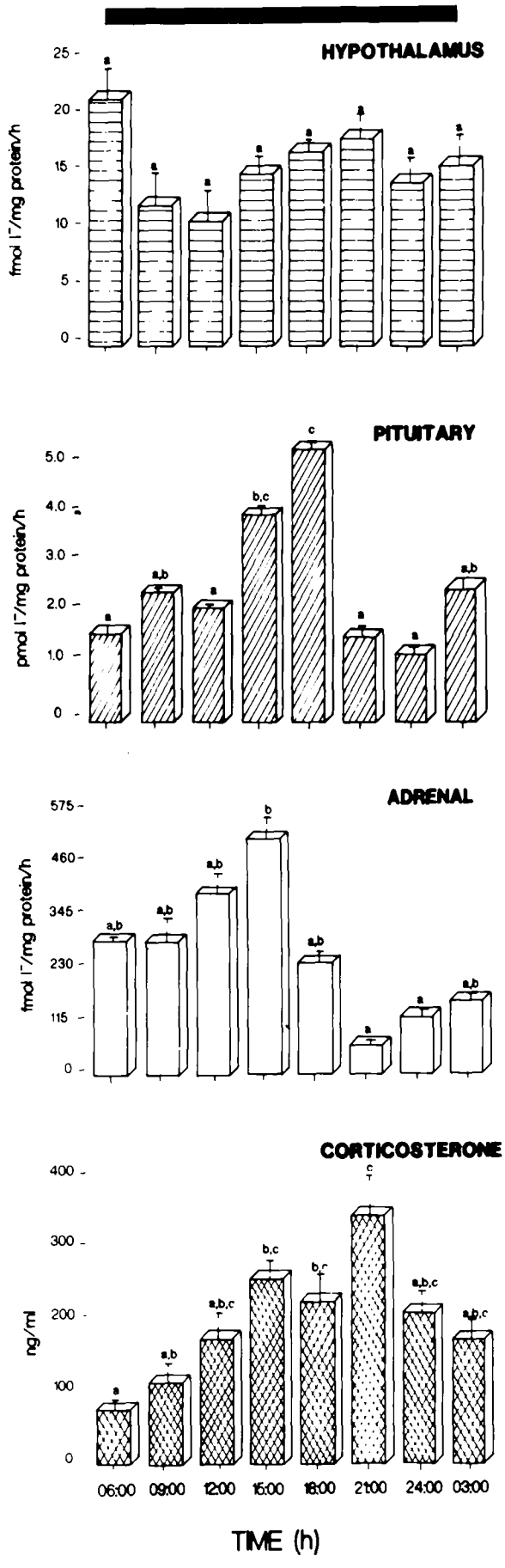

Figure 2 Circadian rhythm of HP, PIT and AG 5'D-II activity and circulating corticosterone levels in rats maintained under continuous darkness during four consecutive days. Each bar represents the mean ± S.E. of enzyme activity quantified in five rats per group from three separate experiments. Means bearing different superscript letters are significantly different. (one-way ANOVA)

Animal housing and sample collection

Normal male Wistar rats weighing 250–300 g, and born in our animal facilities were used. They were housed three per cage in a temperature-controlled room (22 ± 1°C), and were provided with water and standard rat pellets (Purina Lab Chow) *ad libitum*. Animals were exposed to two different photoperiod regimes: (a) to their habitual automatically regulated light/dark cycle of 12:12 (lights on 06:60–18:00 h), or (b) to a continuous darkness regime during four consecutive days. The experimental groups consisted of five animals and preformed three independent experiments along a period of 3–4 month (February to May). At the indicated times (see Results) all animals were sacrificed by decapitation for blood and tissue collection. Animals exposed to continuous darkness were decapitated under dim red light. Trunk blood for corticosterone quantitation was collected into plastic tubes and serum was stored (−20°C) until hormone levels were measured. Immediately after death, the hypothalamus (HP), the anterior pituitary gland (PIT) and both adrenal glands, (AG) were quickly collected, frozen on acetone/dry ice, and stored at −70°C until assayed for 5'D-II activity.

Analytical procedures

Circulating serum levels of corticosterone were measured by a competitive protein-binding assay, as described elsewhere (Luna *et al.*, 1990). The intra- and interassay coefficients of variation were 8.4% ($n = 5$) and 8.0% ($n = 10$), respectively.

Assay of 5'D-II activity was based on the release of radioiodine as originally outlined by Leonard & Rosenberg (1980). The optimal assay condition has been described by Luna *et al.* (1993, 1995 in press). The 5'D-II activity in AG and PIT was determined at ~ two fold the Km substrate (rT3). Briefly, individual tissues were weighed and homogenized (1:10 wt/vol.) in cold 10 mM HEPES buffer, 1 mM EDTA, 25 mM sucrose, pH 7.5. These homogenates were centrifuged for 15 min at 10 000 *g*. Enzyme activity was assayed in the presence of 1 mM PTU. In the case of the HP enzyme it was measured by incubating aliquots (50 µl) of supernatant (5 mg/ml) in a total volume of 100 µl containing 8 nM ^{125}I-T4 + T3 1 µM (for inhibition of 5D, type III) and 20 mM DTT (Kaplan, 1984). Assay conditions for PIT and AG were the same, except for the substrate, that consisted of an isotopic mixture containing 4 nM ^{125}I- rT3 + 36 nM rT3 (115 µl final vol). All assays were performed at pH 7.5 and the incubation time was 3 h at 37°C. The reaction was ended by adding 50 µl of a cold solution containing 50% normal bovine serum and PTU 10 mM + 350 µl 10% TCA. After centrifugation (3000 r.p.m. × 10 min) the supernatant was decanted onto a column (Dowex-50X) and eluted with 2 ml of 10% acetic acid. The ^{125}I- eluate, which is an index of iodothyronine 5'D activity, was determined in a gamma-spectrometer. Enzymatic activity is expressed as femtomoles^{-1} released per mg of protein/h in HP and AG, whereas PIT values are expressed in picomoles^{-1} released/mg of protein/h. Protein was measured as in Bradford (1976).

Statistical analysis

Results are presented as the mean ± S.E. To evaluate for the effect of time, classical procedures analysis of variance (ANOVA) and multiple comparison Scheffe test were applied. To gain information on timing and extent of predictable change, time-series analysis by the least-squares fit of a 24 h cosine curve (Halberg *et al.*, 1977; Sanchez de la Peña *et al.*, 1983) was performed in order to estimate these circadian rhythm characteristics based on the hypothesis that amplitude (e.g. difference between crest and mesor of a best fitting cosine) is equal to zero, in all the rhythmic series of the 5'D-II activity in all the tissues here examined. Moreover, the peak of a fitted single cosine indicating the highest point in a rhythm (acrophase) and referenced to light onset for a 24 h span; and the rhythm-adjusted average (the midway value between the highest and lowest points of a mathematical model, messor) was used to define a rhythm (i.e. a cosine).

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