Circadian regulation of hydroxyindole-*O*-methyltransferase mRNA in the chicken pineal gland *in vivo* and *in vitro*

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The production of the pineal hormone melatonin displays circadian variations with high levels at night. The last enzyme involved in melatonin biosynthesis is hydroxyindole-*O*-methyltransferase (HIOMT, EC 2.1.1.4). The expression of the mRNA encoding chicken HIOMT was investigated *in vivo* and *in vitro* throughout the light/dark cycle, in constant darkness and with light interruption of the dark phase. The stability of HIOMT mRNA was also examined. A day/night rhythm of HIOMT mRNA levels, with a peak at the midlight phase, was observed *in vivo* as well as *in vitro*. Constant darkness did not

abolish this rhythm *in vivo*. One cycle of the HIOMT mRNA rhythm could be observed in constant darkness *in vitro*. In addition, a stimulatory effect of light on HIOMT mRNA levels during the dark phase could be observed *in vivo* as well as *in vitro*. HIOMT mRNA stability was not affected by light or dark conditions, as demonstrated by chase experiments with actinomycin D. The results indicate that the daily changes in HIOMT mRNA concentration reflect transcriptional regulation by circadian oscillators and photosensory mechanisms that are endogenous to the pineal gland.

INTRODUCTION

Melatonin synthesis in the pineal gland undergoes large changes on a daily basis, with a peak occurring at night [1]. The resulting day/night rhythm in plasma melatonin acts as an internal Zeitgeber for the circadian rhythm of locomotor activity [2,3] and mediates the photoperiodic control of reproduction [4,5]. In the chicken pineal gland, rhythmic melatonin production results from two different regulation systems. One system involves a circadian oscillator located in the basal hypothalamus, which controls melatonin synthesis via the sympathetic innervation [6,7]. The effect of light on this system is mediated by the retina [6,7]. A second rhythm-generating system lies within the pineal gland itself. Indeed, the chicken pineal gland isolated *in vitro* produces a robust day/night rhythm of melatonin synthesis that reflects both the activity of an endogenous oscillator and a direct effect of light on pineal photoreceptors [7–9]. The metabolic pathway to melatonin synthesis is: tryptophan → 5-hydroxytryptophan → serotonin → *N*-acetylserotonin → melatonin. The day/night rhythm of melatonin synthesis is generated at the serotonin acetylation step, by a large night-time increase (20-fold in the chicken pineal gland) in the activity of a specific arylalkylamine-*N*-acetyltransferase (EC 2.3.1.87) [10,11]. Hydroxyindole-*O*-methyltransferase (HIOMT, EC 2.1.1.4), the enzyme that catalyses the final step of melatonin synthesis, displays little day/night change (20% daytime increase in the chicken pineal gland) [12,13]. However, we previously reported day/night changes in HIOMT mRNA concentration in the chicken pineal gland, with a 3-fold increase at midday [14]. The characteristics of this daily regulation suggested that HIOMT mRNA concentration might be controlled by circadian oscillators and therefore might provide an unexpected model system for examining the mechanisms of rhythmic gene expression in the chicken pineal gland. To validate this model system, the present study

sought to provide more detailed evidence of the circadian and light-dependent regulations of HIOMT mRNA concentration *in vivo*. Furthermore experiments were performed *in vitro* to examine whether HIOMT mRNA levels could be controlled at transcriptional level, by circadian oscillators and photoreceptors located in the pineal gland itself.

MATERIALS AND METHODS

Materials

[α -³²P]dCTP (3000 Ci/mmol) and Hyperfilm-MP were obtained from Amersham (Les Ulis, France). Dextran sulphate was obtained from Pharmacia (St. Quentin-Yvelines, France) and nitrocellulose sheet (pore size 0.2 μ m) was from Schleicher and Schuell (Aubervilliers, France). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were from Life Technologies (Cergy Pontoise, France). All other chemicals were of analytical grade from various sources.

Animals

One-day-old chicks (*Gallus domesticus*, Hubbard strain) were obtained from Rumolo Co. (Quincy, France). For experiments *in vivo*, chickens were maintained on a 12L:12D light/dark (L/D) cycle for 2 weeks before use. Lights were turned on at 06:00 h and off at 18:00 h clock time, equivalent to Zeitgeber time (ZT) 0 and ZT 12 by convention. To study the daily changes in HIOMT mRNA levels, chickens ($n = 5$ –10 for each group) were killed at intervals of 4 h throughout the light/dark cycle, or throughout constant darkness (D/D). To analyse the effect of light on HIOMT mRNA concentration during the dark period,

Abbreviations used: D/D, constant darkness; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HIOMT, hydroxyindole-*O*-methyltransferase; L/D, light/dark cycle; SSC, 1.5 M NaCl/0.15 M sodium citrate; ZT, Zeitgeber time.

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chickens (10 animals per group) were killed at the midlight phase as a normal lighting control and at the mid-dark phase after 1, 2, 4 or 6 h of light exposure. Pineal glands were removed immediately after the birds had been decapitated; they were frozen in liquid nitrogen and stored at -80°C . Pineal glands from time points during the night were collected under dim red light.

Organ culture

One-day-old chicks were kept in a 12L:12D cycle for 4 days. Pineal glands were collected during the morning of the fourth day and placed in 24-well culture plates (one gland per well), with 200 μl of DMEM supplemented with 2 mM glutamine, 100 i.u./ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 10% FCS. Cultures were incubated (37°C , 5% CO_2) successively on a 12L:12D cycle for 3 days and for 3 days in D/D. Pineal glands ($n = 6-10$) were removed every 4 h, throughout the third L/D cycle and every 2 or 4 h throughout the next three D/D cycles, as indicated in the figure legends. To study the stability of high and low HIOMT mRNA levels, continuing transcription was blocked by the addition of actinomycin D (10 $\mu\text{g}/\text{ml}$) at midday or midnight of the third L/D cycle. Pineal glands were removed after 0, 2, 4, 8 or 12 h of treatment with actinomycin D. The effect of light on the concentration of HIOMT mRNA during the dark phase was also investigated *in vitro*, with 1, 2, 4 or 6 h of light exposure before collecting pineal glands ($n = 10$) at the mid-dark phase. At the times indicated in the Figure legends, pineal glands were removed and frozen in liquid nitrogen. All samples from a time point in darkness were collected under dim red light.

Isolation of total RNA

For experiments *in vivo* as well as *in vitro*, isolation of total RNA was performed as previously described [15]. Each pool of pineal glands ($n = 5-10$) was homogenized by rapid sonication (30 s) in 500 μl of ice-cold LiCl/urea solution (3 M LiCl, 6 M urea) and incubated overnight at 4°C . Total RNA was obtained by centrifugation for 30 min at 4°C and 6000 g. Pellets were sheared by being squirted through 21-gauge needles several times and centrifuged again. Pellets were resuspended in TE/SDS buffer [10 mM Tris/HCl (pH 7.4)/1 mM EDTA (pH 8)/0.5% SDS], extracted twice with phenol and twice with chloroform, and precipitated with ethanol from 0.3 M sodium acetate, pH 6. The precipitates were resuspended in sterile water and the concentration of RNA was determined spectrophotometrically.

Northern blot analysis

Total RNA was denatured with 48% (w/v) formamide and 6.4% (v/v) formaldehyde at 95°C for 2 min, and separated by size in 1% (w/v) agarose denaturing formaldehyde gels containing 0.7 $\mu\text{g}/\text{ml}$ ethidium bromide. After electrophoresis, the RNA was blotted overnight in $10\times\text{SSC}$ (1.5 M NaCl/0.15 M sodium citrate, adjusted to pH 7 with HCl) on nitrocellulose sheets. Blot membranes were dried at 80°C and non-specific sites were blocked for at least 2 h at 42°C with hybridization buffer containing 40% formamide, 10% dextran sulphate, $4\times\text{SSC}$, $1\times\text{Denhardt's}$ solution [where $100\times\text{Denhardt's}$ is 2% (w/v) polyvinylpyrrolidone, 2% (w/v) BSA and 2% (w/v) Ficoll 400], 20 mM Tris/HCl, pH 7.4, and 0.3 mg/ml denatured salmon sperm DNA. Membranes were successively hybridized with the HIOMT cDNA probe, which covered the 1038-base coding sequence and 396 bases in the 3' untranslated region of the chicken pineal HIOMT mRNA [16], and with the human β -actin cDNA probe, obtained by PCR amplification of lymphocyte

cDNA [17], which covered a 207-base sequence of exons 4 and 5. The HIOMT and the β -actin cDNA probes were labelled by random priming [18], with [α - ^{32}P]-dCTP (3000 Ci/mmol). Hybridizations were performed for 16–20 h at 42°C with 100 ng of labelled probe in 5–10 ml of hybridization buffer. Blot membranes were then washed (twice, 30 min each) in $2\times\text{SSC}/0.1\%$ SDS at room temperature, and in $0.1\times\text{SSC}/0.1\%$ SDS at 52°C (twice, 45 min each). The membranes were briefly dried and exposed to Hyperfilm-MP at -80°C with intensifying screens. The autoradiographic intensities of the bands (mean density times surface area) were measured on a Microvision image analyser. The response curve of the film to radioactive standards was used to verify that all density measurements fell in the quasi-linear portion of the sigmoid curve.

RESULTS

Daily rhythm of pineal HIOMT mRNA

In vivo

Northern blot analysis of pineal RNA from chickens kept on a light/dark cycle revealed a diurnal rhythm of HIOMT mRNA levels with a peak at the midlight phase (ZT 6) and a trough at the mid-dark phase (ZT 18) (Figure 1a). Actin mRNA concentration did not vary consistently with the light/dark cycle and

Figure 1 Daily rhythm of chicken pineal HIOMT mRNA level

(a, b) *In vivo*: chicks maintained for 2 weeks under 12L/12D (lights on at ZT 0, lights off at ZT 12) were killed at 4 h intervals throughout the light/dark cycle (five animals at each time point). Total RNA was extracted from pools of five pineal glands and analysed in duplicates on agarose/formaldehyde gel electrophoresis (3 μg of RNA per lane). Northern blot hybridization was performed successively with ^{32}P -labelled cDNA probes to chicken HIOMT and to human β -actin. Densitometric measurement of the radiolabelled bands (mean density times surface area) was performed on a Microvision image analyser, and the HIOMT signal was normalized to the actin signal to allow comparison between the lanes. (c, d) *In vitro*: 42 three-day-old chicks raised in 12L/12D were killed at the end of daytime and the pineal glands were placed in organ culture on 200 μl of DMEM containing 10% (v/v) FCS. On the third day of culture in 12L/12D, the pineal glands were collected at the indicated time points (seven pineal glands per group). Total RNA was extracted from pools of seven pineal glands and analysed in duplicates, as described above. The results presented in (b) and (d) (means $\pm$ S.D., $n = 2$) are HIOMT/actin intensity ratios relative to the value observed at ZT 18.

Table 1 Variations in HIOMT mRNA levels between the mid-dark and midlight phases

Experiment no.	HIOMT/actin mRNA ratios					
	<i>In vivo</i>			<i>In vitro</i>		
	Mid-dark	Midlight	Fold increase	Mid-dark	Midlight	Fold increase
I	0.53	1.39	2.62	2.35	6.04	2.57
II	0.15	0.40	2.67	0.64	1.42	2.22
III	0.25	1.20	4.80	0.43	1.16	2.69
IV	0.24	0.96	4.00	0.80	2.13	2.66
V	0.13	0.44	3.38	2.03	4.42	2.18
VI	1.30	4.26	3.28	0.12	0.27	2.25
VII	—	—	—	0.86	2.18	2.53

was therefore used as an internal standard for densitometric analysis of the autoradiograms (Figure 1b). Six independent experiments comparing the midlight and mid-dark points gave an average 3-fold difference in HIOMT mRNA levels (Table 1).

In vitro

A day/night rhythm in chicken pineal HIOMT mRNA content was also clearly evident *in vitro* on the third day of organ culture (Figure 1c). High values were observed 2 h before light onset and in the first half of the light period, whereas low values were

observed 2 h before light offset and in the first half of the dark period (Figure 1d). Seven independent experiments comparing the midday and midnight points gave an average 2.5-fold difference in HIOMT mRNA levels (Table 1).

Circadian rhythm of pineal HIOMT mRNA

In vivo

Northern blot analysis of pineal RNA prepared at 4 h intervals from chickens housed in constant darkness revealed a circadian HIOMT mRNA rhythm that persisted through at least two cycles (Figure 2a). The period of the rhythm expressed in constant darkness remained close to 24 h. However, it seemed slightly phase-advanced, with the highest values at ZT 2 and lowest values at ZT 14 and ZT 18 (Figure 2b). The amplitude of the rhythm remained in the 2–3-fold range (Figure 2b).

In vitro

Experiments *in vitro* in constant dark conditions revealed a spontaneous rise and fall in HIOMT mRNA during the first subjective day, followed by a second rise and a progressive damping (Figure 2c). The first rise and fall during constant darkness seemed to be phase-advanced, with a peak at ZT 2 and a trough at ZT 10. To confirm this point, the pattern of the HIOMT mRNA oscillation generated during the first subjective day was analysed more closely in three independent experiments.

Figure 2 Circadian rhythm of chicken pineal HIOMT mRNA level

(a, b) *In vivo*: chicks maintained for 2 weeks in 12L/12D (lights on at ZT 0, lights off at ZT 12) were transferred to constant darkness [black and shaded horizontal bars in (a)] and killed at the indicated time points (10 animals per group). Total RNA was extracted from pools of 10 pineal glands and analysed in duplicates, with chicken HIOMT and human β -actin cDNA probes. Densitometric analysis of the autoradiograms was performed as described in Figure 1. The results presented in (b) (means $\pm$ S.D., $n = 2$) are HIOMT/actin intensity ratios relative to the value observed at ZT 18. (c, d) *In vitro*: 100 three-day-old chicks raised in 12L/12D were killed at the end of daytime and the pineal glands were cultured under the same lighting schedule for 3 days. On the fourth day of culture the pineal glands were placed in constant darkness [black and shaded horizontal bars in (c)] and collected at the indicated time points (six pineal glands per group). Total RNA was pool-extracted and analysed in duplicates, as described in Figure 1. The results presented in (d) (means $\pm$ S.D., $n = 2$) are HIOMT/actin intensity ratios relative to the value observed at ZT 18.

Figure 3 Spontaneous increase of HIOMT mRNA level *in vitro* during the first subjective day

Three-day-old chicks ($n = 42$) raised in 12L/12D were killed at the end of daytime and the pineal glands were cultured under the same lighting schedule for 3 days. On the fourth day of culture the pineal glands were placed in constant darkness [black and shaded horizontal bars in (a)] and collected at the indicated time points (six pineal glands per group). Total RNA was pool-extracted and analysed in duplicates, as described in Figure 1. The results presented in (b) (means $\pm$ S.D., $n = 2$) are HIOMT/actin intensity ratios relative to the value observed at ZT 18.

They all revealed that maximal HIOMT mRNA levels were attained around the expected dark/light transition (ZT 22 and ZT 2) with a 3-fold increase over the ZT 18 value (Figure 3). A partial drop in HIOMT mRNA concentration was observed at later time points in constant darkness (Figure 3).

Effect of light on chicken pineal HIOMT mRNA

In vivo

Chickens were exposed to unexpected light for 1, 2, 4 or 6 h before being killed at the mid-dark phase (ZT 18). There was a requirement for 4 h of light *in vivo* to increase HIOMT mRNA levels significantly during the dark period (Figure 4a). The amplitude of the increase was comparable to that of the midlight peak (Figure 4b).

In vitro

The stimulatory effect of light during the dark phase could also be observed *in vitro*. Pineal glands were exposed to unexpected light for 1, 2, 4 or 6 h before the mid-dark phase (ZT 18) on day 3 of culture. A significant increase in HIOMT mRNA levels (2.5-fold) could be observed after 6 h of light exposure (Figures 4c and 4d).

HIOMT mRNA stability

To examine the possibility that the HIOMT mRNA rhythm might be generated at post-transcriptional level, the stability of

Figure 4 Effect of unexpected light on chicken pineal HIOMT mRNA level

(a, b) *In vivo*: Groups of 10 chicks kept for two weeks in 12L/12D (lights on at ZT 0, lights off at ZT 12) were killed at the mid-dark phase either in darkness (ZT 18), or after 1, 2, 4 or 6 h of exposure to unexpected light (ZT 18 + light). A group of animals was killed at the midlight phase as controls (ZT 6). Extraction and analysis of total RNA were performed in duplicates on each pool of 10 pineal glands. Northern blot hybridization was successively performed with 32 P-labelled cDNA probes to chicken HIOMT and to human β -actin. Densitometric analysis was performed as described in Figure 1. The results presented in (b) (means $\pm$ S.D., $n = 2$) are HIOMT/actin intensity ratios relative to the value observed at ZT 18. (c, d) *In vitro*: 60 three-day-old chicks raised in 12L/12D were killed at the end of daytime and the pineal glands were cultured under the same lighting schedule for 2 days. The pineal glands (10 glands per group) were collected at the mid-dark phase of the third 24 h cycle, either in the dark (ZT 18) or after 1, 2, 4 or 6 h of exposure to unexpected light (ZT 18 + light). A group of pineal glands was collected at the midlight phase as controls (ZT 6). RNA extraction and analysis were performed as described in Figure 1. The results presented in (d) (means $\pm$ S.D., $n = 2$) are HIOMT/actin intensity ratios relative to the value observed at ZT 18.

the transcript was analysed during the ascending and descending phases of the 24 h rhythm. On the third day of organ culture, actinomycin D was added at midlight or at mid-dark and HIOMT mRNA was analysed 2, 4, 8 or 12 h later (Figure 5a). Densitometric measurements normalized to 18 S rRNA fluorescence indicated that the half-life of HIOMT mRNA (approx. 2.5 h) was the same under both conditions (Figure 5b).

DISCUSSION

The major finding of the present study is that the daily changes in HIOMT mRNA levels previously observed in the chick pineal gland [14] involve photoreceptors and circadian oscillators that are endogenous to the gland. A first indication of the circadian control of HIOMT mRNA expression had been obtained in previous experiments *in vivo*, where midday and midnight points only had been examined [14]. However, it was not known whether these two selected time points corresponded to the peak and trough of a daily rhythm, nor how sustained this rhythm would be in constant darkness. The results presented here reveal that the peak and trough of the HIOMT mRNA rhythm are indeed at midday and at midnight respectively. Moreover this rhythm was still present when chicks were kept in constant darkness, and there was no evident damping over two cycles.

Figure 5 Chicken HIOMT mRNA stability *in vitro*

Three-day-old chicks ($n = 80$) raised in 12L/12D (lights on at ZT 0, lights off at ZT 12) were killed at the end of daytime and the pineal glands were cultured under the same lighting schedule for 2 days. On the third day of organ culture, actinomycin D (Act-D; $10 \mu\text{g/ml}$) was added to the medium either at the midlight phase (ZT 6) or at the mid-dark phase (ZT 18). Pineal glands were collected after 0, 2, 4, 8 or 12 h of actinomycin D treatment (eight pineal glands per group). Extraction and analysis of total RNA were performed in duplicates on each pineal pool. Densitometric measurements of HIOMT mRNA on autoradiograms were normalized to 18 S rRNA fluorescence. Results in (b) are plotted as means $\pm$ S. D. ($n = 2$). Symbols: □, actinomycin D treatment started at ZT 6; ■, actinomycin D treatment started at ZT 18.

These results provide further support for the notion that HIOMT mRNA expression is an output of the circadian systems that control pineal function.

Two such systems have been described in the chicken. One system comprises circadian oscillators located in the basal hypothalamus, synchronized by retinal inputs and connected to the pineal gland through sympathetic nerves [6,7]. The other system comprises photoreceptors and circadian oscillators that are endogenous to the pineal gland [7–9]. Therefore the next question to be addressed was whether the isolated pineal gland would generate an autonomous rhythm of HIOMT mRNA expression. The experiments *in vitro* presented here indicate that this is so. A clear-cut rhythm could be observed when chick pineal glands were cultured under a light/dark cycle. In addition, one cycle of the HIOMT mRNA rhythm could still be observed when pineal glands were cultured in constant darkness, thus indicating that it was at least partly due to the activity of the pineal oscillator. Interestingly, the spontaneous increase in HIOMT mRNA levels occurred around ZT 24 and was followed by a partial drop. This suggests that the effect of the endogenous oscillator (around ZT 24) might normally be relayed by a stimulatory effect of light to give a peak of HIOMT mRNA levels at the midlight phase. The direct involvement of pineal photoreceptors in controlling HIOMT mRNA expression could

be deduced from the stimulatory effect of light on cultured pineal glands. Previous studies have identified key elements of the phototransduction cascade in chick pineal cells, including a specific photopigment [19,20], transducin [21] and cGMP-activated cationic channels [22]. Further studies are now required to identify the intracellular signals linking phototransduction to stimulation of HIOMT gene expression.

Finally, our chase experiments with actinomycin D revealed an identical half-life of HIOMT transcripts during the ascending and descending phases of the rhythm. These results strongly suggest that the circadian variations in HIOMT mRNA levels do not result from changes in the stability of the transcripts and therefore provide indirect evidence that the rhythm might be generated at the transcriptional level. Together our present results indicate that HIOMT gene expression might constitute a valuable model system to study the mechanism of transcriptional regulation exerted by the circadian oscillator of the chick pineal gland. Other examples of circadian gene transcription in vertebrate tissues cultured *in vitro* comprise the iodopsin gene in chicken retina [23], the tryptophan hydroxylase gene in *Xenopus* retina [24] and the immediate early gene *c-fos* in rat suprachiasmatic nuclei [25]. This wealth of model systems now provides the basis for comparative studies aimed at elucidating the still obscure mechanism of circadian rhythmicity of gene expression in vertebrates. It is not known, for instance, whether these different systems reflect a unique mechanism of circadian gene regulation. Are all these genes effectors of a 'master clock gene' still unidentified in vertebrates, but already described as *frq* in *Neurospora* [26] or as *per* and *tim* in *Drosophila* [27,28]? Comparative studies at promoter level should be informative on this subject as they might indicate whether these different genes have similar upstream regulatory sequences that bind 'circadian transcription factors'. An alternative model of circadian rhythmicity might involve a negative feedback loop between two cAMP-dependent transcription factors, cAMP responsive element binding protein and inducible cAMP early repressor [29]. This model might be of special interest in the chicken pineal gland, because previous studies have described day/night changes in cAMP levels in this tissue [30]. Also, according to previous reports, HIOMT activity in the rat pineal gland can be stimulated by isoprenaline, a β -adrenergic agonist that operates through a cAMP-dependent mechanism [31]. It is not known whether HIOMT mRNA levels in the chicken pineal gland are regulated by cAMP. This question should be examined in further studies because it might provide a clue to circadian gene transcription in the pineal gland.

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