Transcriptional regulation of hydroxyindole *O*-methyltransferase in the chicken pineal gland: day/night changes and long-term effects of light and darkness

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The indolic hormone melatonin is produced by the pineal gland according to a daily rhythm. The terminal step of melatonin synthesis is catalysed by hydroxyindole *O*-methyltransferase (HIOMT, EC 2.1.1.4). Adaptation to constant light or darkness modifies HIOMT activity and concentration. Using a cDNA probe encoding HIOMT, we investigated the effect of environmental lighting on HIOMT gene expression in the chicken pineal gland. HIOMT mRNA levels increased by 100% in constant light as compared with constant darkness. In addition, the present study disclosed the existence of a day/night rhythm of

HIOMT gene transcription, with 3-fold higher mRNA levels at midday than at midnight. This transcriptional rhythm was not accompanied by day/night changes in HIOMT concentration, probably due to a slow turnover of this protein. Unexpected darkness did not prevent the daytime rise in HIOMT mRNA levels, whereas unexpected light prevented the night-time fall in HIOMT mRNA levels. Together, the data would suggest that the day/night rhythm of HIOMT gene transcription in the chicken pineal gland involves both a response to light and the activity of a biological oscillator.

INTRODUCTION

Melatonin production in the pineal gland is organized on a daily basis, with a peak occurring at night [1]. The resulting day/night rhythm in plasma melatonin plays a central role in controlling seasonal breeding and circadian activity/rest cycles, in various animal species [2–5]. Environmental light regulates the activity of two pineal enzymes involved in the conversion of 5-hydroxytryptamine (serotonin) into melatonin. A specific arylalkylamine *N*-acetyltransferase (NAT, EC 2.3.1.87) catalyses the production of *N*-acetyl-5-hydroxytryptamine, the direct precursor of melatonin [6,7]. Depending on species, a 2–50-fold night-time increase in NAT activity appears to drive the daily rhythm of melatonin production [8,9]. The regulation of NAT activity has been studied in some detail in rat and chicken, and its night-time increase was shown to require new RNA and protein synthesis [10,11]. However, direct evidence that environmental light controls NAT gene expression has not been obtained so far. The final step of melatonin synthesis is catalysed by hydroxyindole *O*-methyltransferase (HIOMT, EC 2.1.1.4) [12]. Although HIOMT activity shows little day/night change, its regulation has been demonstrated in chickens and rats kept in constant light or constant darkness for 2 weeks [13,14]. In both species, immunological evidence indicates that environmental light regulates the concentration of HIOMT protein in the pineal gland [15,16], a result suggesting that a control of gene expression might be involved. Further studies were thus needed to examine the effects of light on HIOMT gene transcription. To address this question, we have previously isolated a cDNA clone encoding chicken HIOMT [17]. Using this cDNA probe, we now report long-term effects of light on HIOMT mRNA levels in the chicken pineal gland that match the changes observed in HIOMT protein levels. More surprisingly, day/night changes in HIOMT mRNA levels could be observed. These daily changes appear to reflect both a response to light and the activity of a biological oscillator.

EXPERIMENTAL

Animals

One-day-old chicks (Hubbard strain; *Gallus domesticus*) were obtained from Lecorne Co. (Lencloître, France). To study the daily changes in HIOMT mRNA expression, chicks were raised for 14 days in a light/dark cycle (L/D: 12/12; lights on at 06:00 h, 500 lx). On day 15, four experimental groups (each $n = 15$) were constituted as follows: (a) normal light/dark cycle with killing at 12:00 h; (b) 6 h-extended dark phase with killing at 12:00 h; (c) normal light/dark cycle with killing at 24:00 h; (d) 6 h-extended light phase with killing at 24:00 h. To study the long-term effects of light and darkness, chicks were kept in L/D: 12/12 for 7 days, transferred to constant light (500 lx) or to constant darkness (3 lx, red light) for 15 days and killed at 12:00 h ($n = 25$ in each group).

Isolation of total RNA

Isolation of total pineal RNA was performed as described by Chirgwin et al. [18]. The pineal glands from each group were homogenized as a pool (15 or 25 glands) in 16 ml of ice-cold GIT buffer (4 M guanidine isothiocyanate, 25 mM sodium acetate, pH 6, and 80 mM β -mercaptoethanol). RNA extraction and analysis were run in duplicate on each homogenate. Total RNA was obtained by centrifugation at 130000 *g* for 18 h through a 5.7 M CsCl pad. Pellets were resuspended in TE buffer (10 mM Tris, pH 7.4, and 0.1 mM EDTA), extracted with phenol/chloroform and precipitated with ethanol. The precipitates were resuspended in TE buffer and RNA concentration was measured by the A_{260} . Inter- and intra-group variations of RNA recovery were always within 10%.

Northern-blot analysis

Pineal total RNA was analysed on 1% agarose/0.7 M form-

aldehyde gels containing 0.7 µg/ml ethidium bromide and blotted overnight in 10× SSC (1.5 M NaCl/0.15 M sodium citrate, adjusted to pH 7 with HCl) on to nitrocellulose sheets (Schleicher and Schuell). Non-specific sites were blocked for 2 h at 42 °C with hybridization buffer containing 40% formamide, 10% dextran sulphate (Pharmacia), 4× SSC, 1× Denhardt's solution (100× Denhardt's = 2% polyvinylpyrrolidone, 2% BSA and 2% Ficoll 400), 20 mM Tris, pH 7.4, and 0.3 mg/ml salmon sperm DNA. The HIOMT cDNA probe used for hybridization experiments covered the 1038b coding region plus a 396b 3'-non-coding region of chicken HIOMT mRNA, as previously described [17]. The human β -actin cDNA probe, obtained by PCR amplification of lymphocyte cDNA [19], covered a 207b sequence of exons 4 and 5. The probes were labelled by random priming [20] with either dATP[α^{35} S] (1000 Ci/mmol; Amersham) for HIOMT cDNA or [32 P]dCTP (3000 Ci/mmol; Dositek) for actin cDNA. Hybridization was performed overnight at 42 °C in hybridization buffer. The nitrocellulose sheets were then washed in 2× SSC/0.1% SDS at room temperature (3×15 min) and in 0.1× SSC/0.1% SDS at 52 °C (2×30 min). Autoradiography was performed on Hyperfilm-MP (Amersham) with intensifying screens at -80 °C, for the times indicated in the legends. The autoradiographic intensities of the bands (mean optical density × surface area) were measured on a Biocom 200 image analyser. The response curve of the film to radioactive standards was used to verify that all optical-density measurements fell in the quasi-linear portion of the sigmoid.

Immunotitration of HIOMT activity

Pineal glands (10 from each experimental group) were pooled and homogenized in 1 ml of 50 mM sodium phosphate buffer, pH 7.9, and centrifuged at 13000 *g* for 2 min. Samples of supernatants (10 µl, containing 20 µg of protein) were mixed with increasing amounts (0–30 µl) of anti-HIOMT antiserum [21] and made up to 50 µl with preimmune serum. After incubation of the mixture at 20 °C for 90 min and overnight at 4 °C, the immunoprecipitates were pelleted at 13000 *g* for 10 min and the remaining HIOMT activity of the supernatant was measured by the formation of [3 H]melatonin from *N*-acetyl-5-hydroxytryptamine and [*methyl*- 3 H]-*S*-adenosylmethionine (85 Ci/mmol; NEN), as previously described [21].

RESULTS

Long-term effects of light or darkness on HIOMT mRNA levels

Pineal total RNA extracts from constant-light- or constant-dark-adapted chickens were analysed by hybridization of HIOMT cDNA on Northern blots. Both experimental groups displayed a single HIOMT mRNA transcript of 1.8 kb (Figure 1). Densitometric analysis of the autoradiograms indicated that constant light exposure increased HIOMT mRNA concentration 2-fold, as compared with constant darkness (Figure 1). Equal sample loading and transfer efficiency were verified by ethidium bromide-induced RNA fluorescence (results not shown). Similar results were obtained in two separate experiments.

Daily changes in HIOMT gene transcription

When chickens were kept in a L/D:12/12 lighting regime, HIOMT mRNA levels increased during the daytime, whereas actin mRNA levels remained unchanged throughout the light/dark cycle (Figure 2). With actin mRNA as an internal standard,

Figure 1 Long-term effects of light or darkness on HIOMT mRNA levels

Chicks kept for 1 week in L/D:12/12 were transferred to constant light (LL) or constant darkness (DD) for 2 weeks and killed at midday. In each group, 25 pineal glands were pooled and homogenized, and total RNA was extracted and analysed in duplicates from each homogenate. Northern-blot hybridization was performed with 35 S-labelled HIOMT cDNA. An autoradiogram of duplicate extracts is presented (1 µg of total RNA per lane, 96 h exposure time).

Figure 2 Daily variations of HIOMT mRNA levels

Chicks raised for 2 weeks in L/D:12/12 were killed at midday or at midnight, and the pineal glands from each group ($n = 15$) were pooled and homogenized. Extraction and analysis of total RNA were performed in duplicates for each experimental group. The Northern blots were probed successively with 35 S-labelled HIOMT cDNA and with 32 P-labelled actin cDNA. A representative autoradiogram of duplicate samples is shown (2 µg of total RNA per lane, 72 h exposure time). The HIOMT/actin intensity ratios are displayed below each lane.

Figure 3 Immunotitration of midday (□) and midnight (■) HIOMT activity

Chicks raised for 2 weeks in L/D:12/12 were killed at midday or at midnight. In each group, 10 pineal glands were pooled and homogenized, and samples (20 μg of protein in 10 μl) were mixed with increasing quantities (0–30 μl) of anti-HIOMT antiserum. The volume was made up to 50 μl with preimmune serum. After incubating the mixture at 20 °C for 90 min and overnight at 4 °C, the immunoprecipitates were pelleted at 13000 *g* for 10 min and the supernatants were assayed for HIOMT activity. Results are duplicate measurements; the S.E. was within the symbol size.

Figure 4 Effects of unexpected periods of light or darkness on HIOMT mRNA levels

Chicks kept for 2 weeks in L/D:12/12 were divided in four experimental groups: a, normal light/dark cycle with killing at midday; b, 6 h-extended dark phase with killing at midday; c, normal light/dark cycle with killing at midnight; d, 6 h-extended light phase with killing at midnight. In each group, 15 pineal glands were homogenized as a pool. Extraction and analysis of total RNA were performed in duplicates on each pool. The Northern blots were probed successively with ³⁵S-labelled HIOMT cDNA and with ³²P-labelled actin cDNA. The autoradiogram shows only one replicate of each group (2 μg of total RNA per lane, 168 h exposure). The values indicated below each lane are the means of duplicate samples (S.D. within 5%).

densitometric analysis of the autoradiograms from three separate experiments revealed that HIOMT mRNA concentration was 3–4-fold higher at midday than at midnight (Figure 2). In contrast, HIOMT activity was only 20% higher at midday than at midnight (Figure 3). We verified by immunotitration of enzyme activity that the day/night change in HIOMT concentration was also within 20% (Figure 3).

Effects of unexpected periods of light and darkness

The daily changes in HIOMT mRNA content might be directly driven by the light/dark cycle or controlled by a circadian oscillator. This point was addressed in one experiment, based on the effects of unexpected periods of light or darkness on HIOMT

mRNA levels. As illustrated in Figure 4, HIOMT mRNA levels were high at midday, regardless of the animals being kept in the light (lane a) or in the dark (lane b) during the expected light phase, a result indicating that the daytime rise in HIOMT mRNA levels was not a mere response to light. In contrast, light-sensitivity of HIOMT gene transcription could be demonstrated at night (Figure 4). Indeed, the midnight fall in HIOMT mRNA content, normally observed in the dark (lane c), could be prevented by keeping the animals in the light (lane d). In this experiment, a major HIOMT mRNA transcript of 1.8 kb (90% of the signal) and a minor one of 4 kb (10% of the signal) could be observed (Figure 4). Both signals displayed similar changes in response to light. Accordingly, the sum of the 1.8 kb and 4 kb transcripts was taken as representative of HIOMT gene expression.

DISCUSSION

The present study provides the first evidence that environmental light regulates HIOMT gene transcription in the pineal gland. Long-term exposure to continuous light increased HIOMT mRNA levels 2-fold, as compared with continuous darkness. Regulation of HIOMT gene transcription may thus account for the 2-fold difference in HIOMT activity and concentration previously reported between constant-light- and constant-dark-adapted chickens [13,16]. Furthermore, the present study disclosed that HIOMT gene transcription is organized on a daily basis, with higher mRNA levels during the daytime. This result was unexpected, as it is well established that HIOMT activity displays at most a 20% daytime increase in the chicken pineal gland [22]. Immunotitration experiments revealed no day/night difference in the intrinsic activity of the enzyme. So the small amplitude changes in HIOMT activity do not result from the damping of the larger mRNA rhythm by an inverse cycle of post-translational activation. Further experiments would be required to determine whether the translation efficacy of HIOMT mRNA may be lower at midday than at midnight. More conceivably, the discrepancy observed between the rhythmic expression of HIOMT mRNA and the nearly constant level of the enzyme could be explained by a slow turnover of the HIOMT protein. Consonant with this assumption, we have observed that complete blockade of protein synthesis by cycloheximide in cultured chick pineal cells did not modify HIOMT activity after 24 h (M. Bernard and P. Voisin, unpublished work). Previous studies have also indicated that HIOMT is a relatively stable protein in homogenates [23]. Regulation of gene transcription and slow turnover of the HIOMT protein would explain the long-term changes in HIOMT activity, evoked by constant light or constant darkness [16]. This regulation system would appear well adapted to produce seasonal changes in HIOMT expression, in response to the changes in day length. However attractive, this hypothesis is not supported by previous studies in sparrows, showing that HIOMT activity increases during the winter [24]. A more thorough analysis of the seasonal changes in HIOMT gene expression, from the transcriptional level down to the active enzyme, might provide information of general interest on the molecular mechanisms of seasonality. Further studies are also required to elucidate the mechanism of the day/night rhythm of HIOMT gene transcription at different levels of organization (i.e. organism, cell, gene promoter). One piece of information obtained in the present study is that HIOMT gene transcription is light-sensitive during the night, but not during the daytime. Because the daytime increase in HIOMT mRNA levels can be observed in the absence of light, a circadian oscillator may be involved in controlling the daily rhythm of HIOMT gene

transcription. At this point, it is not known whether the effect of light on HIOMT mRNA levels is exerted directly on the pineal photosensitive cells described in the chicken [25], or through the retina and a neuronal pathway. Similarly, the daily rhythm of HIOMT gene transcription might be generated by the circadian oscillator of the pineal gland that also controls NAT activity [25], or by the hypothalamic pacemaker that delivers a rhythmic input to the pineal gland [26]. Further studies on pineal cell cultures should help to address these questions and to identify extra- and intra-cellular signals that regulate HIOMT mRNA levels. Because the day/night rhythm of melatonin synthesis mostly relies on rapid changes in NAT activity, this enzyme has attracted a large number of studies concerned with the photoperiodic regulation of pineal function. However, there has been no direct evidence that daily changes in NAT activity reflect a regulation of NAT gene expression. The day/night rhythm of HIOMT mRNA levels described herein provides an unexpected possibility to study the photoperiodic control of gene expression in the pineal gland.

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