Relationship of atypical melatonin rhythm with two circadian clock outputs in B6D2F1 mice

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Li, Xiao-Mei, Xu-Hui Liu, Elisabeth Filipski, Gérard Metzger, Philippe Delagrange, Jean-Philippe Jeanniot, and Francis Lévi. Relationship of atypical melatonin rhythm with two circadian clock outputs in B6D2F1 mice. Am J Physiol Regulatory Integrative Comp Physiol 278: R924–R930, 2000.—Circadian rhythms in body temperature, locomotor activity, and the circadian changes of plasma and pineal melatonin content were investigated in B6D2F1 mice synchronized by 12 h of light and 12 h of darkness. During 8 wk continuous recording, activity and temperature displayed a marked stable and reproducible circadian rhythm, with both peaks occurring near the middle of darkness. Both 24- and 12-h rhythmic components were also significantly detected. Mean plasma melatonin concentration rose steadily during the light span and reached a maximum (30.6 ± 10.0 pg/ml) at 11 h after light onset (HALO), then gradually decreased after the onset of darkness to a nadir (4.7 ± 0.4 pg/ml) at 20 HALO. Mean pineal content followed a pattern parallel to that of plasma concentration (peak at 11 HALO: 17.7 ± 1.0 pg/gland; trough at 17 HALO: 4.7 ± 1.0 pg/gland). In addition, a second sharp peak was observed at 21 HALO (20.2 ± 3.5 pg/gland). Plasma and pineal contents displayed large and statistically significant circadian changes, with a composite rhythm of period (24 ± 12 h). This mouse model has predominant production and secretion of melatonin during the day. This possibly contributes to a similar coupling between chronopharmacology mechanisms and the rest-activity cycle in these mice and in human subjects.

circadian rhythms; body temperature; locomotor activity; pineal gland

THE REST-ACTIVITY CYCLE AND the nocturnal secretion of melatonin by the pineal gland are controlled by suprachiasmatic nucleus (SCN) function in nocturnally active rodents. Thus stereotaxic destruction of SCN suppressed these rhythms in rats or hamsters. These rhythms were restored after SCN transplantation (16, 17, 22). The endogenous circadian periodicity usually differs from precisely 24 h, but the regular alternation of light and darkness over 24 h sets the endogenous period to precisely this value. Because melatonin is mostly secreted at night in mammals, its physiological peak occurs during the activity span of nocturnally active rats or hamsters and during the rest span of diurnally active humans (1, 4). Although mice are also nocturnally active, their ability to secrete melatonin has been challenged (11, 12). Part of this controversy stemmed from the use of nonspecific methods, using radiolabeled antibodies with unknown specificity and/or insufficient assay sensitivity. Indeed, the use of gas chromatography (GC)-mass spectrometry (MS) was needed to demonstrate melatonin in the pineal gland of C57BL/6 or BALB/c mice, which were previously deemed to lack any melatonin secretion (28).

On the other hand, male DBA/2 × female C57BL/6 hybrids, so called B6D2F1 mice, have been extensively used for investigating the chronopharmacology of anticancer drugs as an experimental prerequisite to the clinical testing of treatment schedules adapted to circadian rhythms in cancer patients (14, 19). Several cellular or metabolic rhythms responsible for anticancer drug chronopharmacology displayed a similar phase relationship with the rest-activity cycle both in these mice and in cancer patients. These results led to the hypothesis of a coupling between chronopharmacology mechanisms and the rest-activity cycle across species (18, 19). In addition, it has been reported that administration of melatonin modified circadian activity rhythms in mice and in rats (2, 8, 10, 20). Therefore, we first investigated the rest activity and body temperature rhythms in B6D2F1 mice and then studied the circadian changes of melatonin contents in plasma and in pineal gland.

MATERIAL AND METHODS

Animals and study designs. All the experiments involved male B6D2F1 mice (male DBA/2 × female C57BL6/6) bred by Institut Francais Fievre Aphteuse-Centre Recherche Elevage Des Oncins (L’Arbresle, France). Animals had free access to water and food (A04–10, UAR, Villemonois-Sur-Orge, France) and were housed one or two per cage, according to the experiment. All the mice were kept in an autonomous chronobiological facility (ESI Flufrance, Arcueil, France). Each facility is equipped with temperature control (23 ± 1°C) and comprises four or six compartments, each one being

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provided with separate filtered air (100 ± 10 l/min) and lighting regimen. Light intensity at cage level ranged from 223 to 315 lux. All the manipulations during the dark phase were performed under dim red light (7 lux).

Experiment 1 studied the rhythms in body temperature and rest activity in mice kept in a 12:12-h light-dark cycle. Experiments 2–5 investigated melatonin rhythms in plasma and pineal gland in animals synchronized with a 12:12-h light-dark cycle for 3 wk before the study (Table 1).

Experiment 1. Eight 9-wk-old mice were implanted with an intraperitoneal sensor (PhysioTel, TA10TA-F20, Data Sciences, St. Paul, MN) on arrival. They were singly housed and synchronized with a 12:12-h light-dark cycle, with light from 0600 to 1800 for 8 wk. Rest activity and body temperature were automatically recorded every 10 min throughout the entire study duration in each animal.

Experiments 2–4. Mice were received at 6 wk of age and were synchronized with a 12:12-h light-dark cycle. They were housed two per cage on arrival and were randomized to one of six groups in experiment 2, one of twelve groups in experiment 3, and one of two groups in experiment 4. Each group corresponded to a different circadian time to be studied and was kept in a different compartment of a chronobiological animal facility. The time of light onset in each compartment was staggered by 4 h in experiment 2 (i.e., 0600 for group 1, 1000 for group 2, 1400 for group 3, etc.), by 2 h in experiment 3 and by 12 h in experiment 4.

Six circadian stages were studied in experiment 2, 12 in experiment 3, and two in experiment 4. These circadian stages were expressed in hours after light onset (HALO). Six mice were studied at each time point in each experiment.

After a 3-wk synchronization of the mice, rectal temperature was measured in each mouse before blood sampling from the retroorbital sinus (0.7 ml). Blood was collected into a heparinized tube. Plasma was immediately separated by centrifugation at 3,000 g for 25 min at 4°C, then stored at −80°C until melatonin determination with RIA in experiments 2 and 3 or with GC-MS in experiment 4.

Experiment 5. Synchronization and study design were similar to that of experiment 3, with 12 circadian stages explored, each one being separated from the next by 2 h. Six mice were killed by cervical dislocation at each time point.

After death, the pineal glands attached to the skull caps were cut away and immediately frozen in liquid nitrogen. Sampling of each pineal was performed within 1 min. Samples were stored at −20°C until homogenization. Before the assay, each pineal gland was homogenized for 30 s at 4°C using a sonicator (model 250/450 sonifier, Maurepas, France) in 350 µl of the assay buffer (pH 6.0; 0.1 M tricine; 154 mM 0.9% NaCl; 0.1% gelatin). Samples were centrifuged at 15,000 g for 5 min, and the supernatant was removed and stored at −20°C until melatonin determination by GC-MS.

RIA. Melatonin was measured by RIA using a specific rabbit antiserum antimelatonin (from Stockgrand) at a final dilution of 1:49,500 and [2-125I]iodomelatonin as radioligand. The method involved a liquid-liquid extraction with chloroform before the competition step in a tricine buffer.

With the use of 100 µl in duplicate, the limit of detection of the method was 4 pg/ml. Intra- and interassay precisions were <15.2% and 11.2%, respectively. The specificity of the antibodies used was high because the percentage of cross-reactivity was 5.3% for 6-hydroxymelatonin and 0.23% for 6-sulfatoxymelatonin, the two main metabolites of melatonin.

GC-MS. After extraction with dichloromethane, melatonin and its internal standard (heptadeuterated melatonin) are taken to dryness then derivatized with pentafluoropropionic anhydride in ethyl acetate. The derivatives are separated on a GC column and detected by their molecular ions. The method is linear within the calibration range from 1 to 100 pg/ml using 1 ml of biological sample. The method is highly specific, precise, and accurate (coefficient of variation and relative error <10% for both intra- and interassays) (27).

Statistical analyses. Seven-day time series of temperature and activity from each mouse were analyzed by two methods using Dataquest III software (9). First, power spectrum analysis (Fourier transform) was applied to 30-min average data intervals. Least-square cosine regression (cosinor) was then applied for test periods, differing by 15 min, within the range of the dominant period ± 1 h. The period that corresponded to the highest percent rhythm (proportional to highest amplitude) was considered as the dominant one, if P < 0.05.

Time series of each variable were further analyzed for 24- and 12-h rhythmicities with the cosinor method (21). A rhythm was characterized with three parameters: the mesor (rhythm-adjusted mean), the amplitude (one-half the difference between minimum and maximum of best fitting cosine function), and the acrophase (time of maximum in best fitting cosine function, with light onset as phase reference). If a rhythm was detected, the three parameters were computed with their respective 95% confidence limits. This was achieved when amplitude differed from zero (non-null amplitude F-test with P < 0.05).

Means ± SE were computed for each variable and/or time point. The statistical significance of differences between time points was validated by one-way ANOVA. The statistical significance of both conventional and chronobiological statistics was needed to qualify temporal changes as rhythms.

Table 1. Characteristics of the experiments involving a total of 200 B6D2F1 mice synchronized with 12:12-h light–dark cycle

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Number of Mice</th>
<th>End Point</th>
<th>Method</th>
<th>Sampling Frequency and Observation Span</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>Rest-activity body temperature</td>
<td>Intraplottone sensor monitoring (Dataquest III)</td>
<td>every 10 min for 8 wk</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>Plasma (melatonin)</td>
<td>RIA</td>
<td>every 4 h for 24 h (0, 4, 8, 12, 16, or 20 HALO)</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>Plasma (melatonin)</td>
<td>GC-MS</td>
<td>every 2 h for 24 h (1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23 HALO)</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>Plasma (melatonin)</td>
<td>GC-MS</td>
<td>every 2 h for 24 h (1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23 HALO)</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>Pinene melatonin content</td>
<td>GC-MS</td>
<td>every 2 h for 24 h (1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, or 23 HALO)</td>
</tr>
</tbody>
</table>

HALO, hours after light onset; GC, gas chromatograph; MS, mass spectrometry.
RESULTS

Body temperature and locomotor activity rhythms. In the 12:12-h light-dark cycle condition, a 24-h rhythm in body temperature and in locomotor activity was found for each mouse. During 8 wk of continuous recording, activity and temperature displayed a marked stable and reproducible circadian rhythm (Fig. 1). The dominant period of temperature and activity rhythms of each mouse was ~24 h according to power spectrum and cosinor analysis. A 12-h rhythmic component was also validated. The acrophases occurred near the middle of darkness for both rhythms (Table 2).

Plasma melatonin rhythm. The 24-h mean plasma concentration of melatonin was similar in each experiment, despite different assay methods used for experiments 2-4. Thus mean melatonin concentration (±SE) was 11.3 ± 1.2 pg/ml in experiment 2, 14.5 ± 1.5 pg/ml in experiment 3, and 10.5 ± 3.0 pg/ml in experiment 4 (average of 3 experiments: 13.2 ± 1.0 pg/ml).

Mean plasma melatonin concentration measured by RIA varied according to sampling time in experiments 2 and 3, from 7 pg/ml in the late dark or early light span up to 18–30 pg/ml in the late light span. Quite strikingly, melatonin plasma level fell abruptly within 3 h.
after the onset of darkness (Fig. 2). A circadian rhythm was statistically validated with ANOVA and cosinor. Moreover, a 12-h rhythmic component was also statistically validated with cosinor analysis in experiment 3, which had been especially designed for testing this possibility. Cosinor analysis of pooled data from both experiments demonstrated 24- and 12-h rhythmic components (Table 2).

In experiment 4, mean plasma melatonin concentration (±SE), as measured by GC-MS, also differed significantly as a function of sampling time. It was 9.8 ± 2.1 pg/ml near the expected time of peak (11 HALO), and 1.0 ± 0.1 pg/ml near the expected time of trough (23 HALO; Fig. 2). Furthermore, the figures of melatonin concentration, as measured with GC-MS, were similar to those measured with RIA at the same time points, yet in different experiments. Pineal melatonin rhythm. The 24-h mean melatonin content of pineal gland, as measured with GC-MS, was 12.1 ± 0.7 pg/gland. Mean pineal melatonin content also varied significantly according to sampling time, with a bimodal pattern: a first maximum (17.7 ± 1.0 pg/gland) was encountered near the end of the light span, and a second sharp and narrow maximum (20.2 ± 3.5 pg/gland) was found in the second half of darkness (Fig. 3). Mean minimum content was found at 17 HALO (4.7 ± 1.0 pg/gland). Cosinor analysis further documented both 24- and 12-h rhythms, with similar amplitudes relative to mesor. Acrophases of pineal melatonin rhythms were close to those observed for plasma melatonin (Table 2).

![Fig. 2. Circadian variations of plasma melatonin concentration in B6D2F1 mice in 3 separate experiments. Melatonin concentration was measured with RIA (experiments 2 and 3) or with gas chromatograph (GC)-mass spectrometry (MS; experiment 4). Each point represents mean (±SE) of 6 animals. One-way ANOVA revealed a statistically significant difference as a function of circadian time (F = 6.0, 4.1, and 76.1 for experiments 2, 3 and 4, respectively, with P = 0.001). Circadian rhythms were further validated by cosinor for experiments 2 and 3 (P < 0.0001). A 12-h rhythm was apparent and statistically validated with cosinor for experiment 3 (P = 0.03).](http://ajpregu.physiology.org/fig/3)

![Fig. 3. Circadian variations of pineal melatonin content as measured by GC-MS in B6D2F1 mice (experiment 5). Open box indicates duration of light, and black box indicates duration of darkness. Each point represents mean (±SE) of 6 animals. Differences according to sampling time were statistically validated with ANOVA (F = 10.2, P < 0.0001). Significant 24- and 12-h rhythms in pineal melatonin contents were statistically validated with cosinor (P = 0.005).](http://ajpregu.physiology.org/fig/4)
DISCUSSION

Mean melatonin plasma concentration varied ~6.5-fold over 24 h in male B6D2F1 mice. Such circadian change was highly reproducible from one study to the next. It was synchronous with a 4.3-fold circadian variation in pineal melatonin content. The data thus support the reflection of the melatonin production rhythm in circulating melatonin levels (23).

The ability of mouse pineal to secrete melatonin was challenged until recently (12, 28). Nevertheless, the use of more specific antibodies for RIA, the confirmation of melatonin in these samples by GC-MS, and high sampling frequency have established that most, if not all, mouse strains produce melatonin (28). We combined these methods and found that melatonin levels rose steadily throughout the light span and reached a circadian maximum 1 h before darkness onset both in the pineal gland and in the plasma of B6D2F1 mice. A sharp second peak in pineal gland melatonin content occurred 3 h before the end of darkness and accounted for a statistically significant 12-h periodic component, which was also detected for plasma melatonin levels.

The nocturnal maximum in pineal melatonin content corresponded to that already reported in several other mouse strains, using RIA and/or GC-MS for melatonin determinations (12, 28). The demonstration of a diurnal maximum was striking and has not been reported in mice, to the best of our knowledge. Most recent studies have examined whether the pineal gland of several mouse strains could produce melatonin. Samples were obtained at high frequency during the dark span (up to every 15 min), because the secretion of this hormone has been associated with darkness both in laboratory rodents and in human beings. The fact that sampling was limited to every 4 h during the light span may have resulted in missing a diurnal melatonin peak (28).

Fig. 4. Circadian changes in body temperature (A) and locomotor activity (B). Each point represents mean of 8 mice monitored for 7 consecutive days during eighth week of 12:12-h light-dark cycle, pineal melatonin content (C, experiment 5), and plasma melatonin concentration (D, experiments 2 and 3). Each point is mean ± SE.
creased liver catabolism of this hormone during darkness, an issue that deserves further exploration.

The regular rise of pineal and plasma melatonin levels during the light span indeed occurred during the rest span of these mice. Thus continuous activity and temperature recording for 8 wk demonstrated that these hybrid mice were nocturnally active and had marked stable and reproducible circadian rhythms in both variables when kept in 12:12-h light-dark conditions. Inspection of individual and average curves indicated that activity level and body temperature started to rise ~2 h before dark onset, plateaued for ~10 h and began to clearly drop 2–4 h before light onset. These circadian stages corresponded to the first and second pineal melatonin peaks, respectively (Fig. 4), which may indicate common regulatory mechanisms of the rhythmic patterns of all three variables. Furthermore, these anticipatory changes, with regard to photoperiodic switches, strongly support the endogeneity of these three rhythms.

The ability of nocturnal melatonin secretion to be suppressed by low-light intensity was demonstrated in humans and in rats as well as in C3H and in CBA mice (5, 11, 15, 26). No such effect was found in the present hybrid mice. Such apparent lack of photosensitivity of pineal melatonin secretion might explain why B6D2F1 mice maintained circadian rhythms in body temperature and locomotor activity, despite exposure to continuous light for up to 19 wk (25). Conversely, continuous light exposure suppressed the circadian rhythms in both body temperature and rest activity of male Sprague-Dawley rats and reduced sixfold mean melatonin plasma level (6, 7).

Taken together, the results indicate that pineal gland photosensitivity can vary from one rodent species or strain to another. Such differences can be associated with species or strain-specific mechanisms of circadian coordination. More specifically, B6D2F1 mice predominantly secrete melatonin during the light span, when they rest, and when their body temperature rhythm is near its low point. This possibly contributes to a similar coupling between chronopharmacology mechanisms and the rest-activity cycle in these mice and in human subjects.

Perspectives

In rats and in several mouse strains, melatonin secretion predominates at night and coincides with maxima in body temperature and in locomotor activity. Despite the fact that B6D2F1 mice are also nocturnally active, melatonin secretion by the pineal gland predominates during day time, i.e., during their rest span. The phase relationship between melatonin secretion and both circadian clock outputs in these hybrids is similar to that found in human beings. This mouse model is valuable for guiding chronopharmacological studies in humans.

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