Rapid phase advance of the 24-h melatonin profile
in response to afternoon dark exposure

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Van Cauter, Eve, Rodrigo Moreno-Reyes, Elif Akseki, Mireille L’Hermite-Balériaux, Ulrich Hirschfeld, Rachel Leproult, and Georges Copinschi. Rapid phase advance of the 24-h melatonin profile in response to afternoon dark exposure. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E48–E54, 1998.—To investigate the adaptation of melatonin secretion to an abrupt time shift and the effects of sleep facilitation with a hypnotic, eight subjects were submitted to an 8-h advance shift achieved by advancing bedtimes from 2300–0700 to 1500–2300. Each subject participated in two studies (i.e., placebo and zolpidem). Each study included a baseline period with dim light during waking hours and 2300–0700 bedtimes in total darkness. Blood samples for determination of plasma melatonin were obtained at 20-min intervals for 68 h. Advanced exposure to sleep and darkness resulted in a nearly 2-h advance of melatonin onset, which appeared within 6 h after lights-out during the first shifted night, and an almost 1-h advance of the melatonin offset. No further adaptation occurred during the second shifted sleep period. Zolpidem had no beneficial effects on the adaptation of the melatonin profile. There was no relationship between sleep parameters and the magnitude of the melatonin shifts. Thus the overall advance of melatonin profiles was primarily achieved during the initial exposure to an 8-h period of darkness. The present data suggest that exposure to dark affects human circadian phase.

sleep; zolpidem; circadian rhythms; light

ADAPTATION OF THE HUMAN CIRCADIAN SYSTEM TO AN ABRUPT PHASE ADVANCE OF SLEEP-WAKE AND DARK-LIGHT CYCLES, AS OCCURS IN EASTWARD JET LAG OR ADVANCE WORK-SHIFT ROTATIONS, APPEARS TO BE MORE DIFFICULT THAN ADAPTATION TO A PHASE DELAY OF SIMILAR MAGNITUDE (3). DIFFICULTIES IN OBTAINING SUFFICIENT AMOUNTS AND QUALITY OF SLEEP AT AN ABNORMAL CIRCADIAN TIME FREQUENTLY RESULT IN THE USE OF HYPNOTICS, BUT IT IS UNCLEAR WHETHER CIRCADIAN PHASE IS AFFECTED BY THIS MANIPULATION OF SLEEP-WAKE HOMEOSTASIS.

The 24-h melatonin profile is a robust marker of circadian phase in humans (16, 20). Because melatonin secretion is not affected by daytime sleep, nocturnal sleep deprivation, or sleep-wake transitions, alterations of melatonin profiles provide reliable estimations of circadian adaptation to phase shifts (1, 2).

The present study was designed to investigate, during the first 40 h after the shift, the adaptation of plasma melatonin profiles to an 8-h advance shift of the dark-light, sleep-wake, and feeding cycles, achieved in a sleep laboratory without enforcing sleep deprivation, and to evaluate the impact of pharmacological sleep facilitation using zolpidem, a widely used nonbenzodiazepine hypnotic, which has no direct effects on melatonin secretion (8).

SUBJECTS AND METHODS

Subjects

Eight normal subjects (4 men and 4 women), 21–33 yr old, were selected after a careful clinical and biological evaluation. All subjects were of normal weight (mean ± SD body mass index: 21.9 ± 0.7 kg/m²). The subjects were nonsmokers, had not taken any drug during the last 6 wk, and had no history of substance abuse. Night and/or shift workers, subjects who had traveled across time zones during the 2 mo preceding the study, and subjects with sleep complaints or with a personal history of endocrine, metabolic, neurological, or psychiatric disorders were excluded. Female subjects had normal ovulatory cycles, had received no oral contraceptives for at least 6 wk before the study, and were investigated at midfollicular phase. The protocol was approved by the Institutional Review Board, and written informed consent was obtained from all subjects.

Experimental Protocol

One week before the start of the investigation, the subjects were required to spend two consecutive nights in the sleep laboratory to habituate to the hospital environment and recording procedures. Subsequently, all subjects participated in three different studies (i.e., placebo, zolpidem, and bright light; see below) separated by at least 1 mo. The order of the studies was randomized. Consistent with the timing and duration of exposure, bright light did not affect markers of circadian phase derived from the melatonin profiles (16a) but had beneficial effects on sleep quality and plasma levels of thyrotropin, which have been previously described (12). Therefore, the present report will focus on the comparison between the placebo and zolpidem studies.

During the week preceding the first study as well as during the periods between the studies, the subjects had to maintain regular sleep-wake cycles (bedtime 2300–0700 in total darkness) and meal schedules (breakfast 0800, lunch 1230, dinner 1900). For each study, the subjects were hospitalized in the sleep laboratory for four consecutive nights. During the first night, which served as an additional habituation night, the subjects remained recumbent in total darkness from 2300 to 0700, and sleep was polygraphically recorded. On awakening, the subjects remained awake in an upright sitting position in dim-light conditions (≤200 lux) until 2300. At 1000, a sterile heparin-lock catheter was inserted in the forearm, and, starting at 1100, 2-ml blood samples were obtained at 20-min intervals for 68 consecutive hours, which included a 28-h baseline period and 40 h of postshift conditions, including two shifted sleep periods. A schematic representation of the study design is given in Fig. 1. Light-dark conditions during the
68-h sampling period are also shown for each study on the horizontal axes of Figs. 2 and 3. During the scheduled sleep/dark periods, the indwelling catheter was connected to plastic tubing extending to an adjoining room.

During baseline conditions, meals were served at 0800, 1230, and 1900, and water was allowed ad libitum. From 2300 to 0700, subjects remained recumbent in total darkness, and sleep was polygraphically recorded. Breakfast and lunch were then served at 0800 and 1230, respectively, and the subjects were maintained awake in dim-light conditions until 1500.

The first shifted period started at 1500; the subjects had to go to bed and remain recumbent in total darkness until 2300 while sleep was polygraphically recorded. At 2300, lights were switched on and the subjects had to get up. Recumbency

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**Fig. 1.** Schematic representation of study design. Black bars represent bedtime/dark periods, and white areas represent periods of wakefulness in dim indoor light. Timings of administration of placebo (P) or zolpidem (Z) are shown by arrows. Numbers represent time, using 24-h time system. L, lunch; D, dinner; B, breakfast.

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**Fig. 2.** Mean ± SE plasma melatonin profiles during placebo (A) and zolpidem (B) studies. Black bars indicate bedtime periods, and hatched bars indicate periods of wakefulness in dim light (≤200 lux). Arrows depict time of administration of placebo or zolpidem. Limit of sensitivity of melatonin assay was 2 pg/ml.

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**Fig. 3.** Individual plasma melatonin profiles in a representative subject during placebo (A) and zolpidem (B) studies. Black bars indicate bedtime periods, and hatched bars indicate periods of wakefulness in dim light (≤200 lux). Arrows depict time of administration of placebo or zolpidem. Limit of sensitivity of melatonin assay was 2 pg/ml.
was not allowed during the scheduled waking periods, and subjects walked around the unit, read, listened to radio, and engaged in conversation with the investigators. Breakfast was served at 0000. Thus the dark-light, sleep-wake, and feeding cycles were abruptly advanced by 8 h. Lunch and dinner were served at 0430 and 1100, respectively, and the subjects were maintained awake until 1500.

The second shifted period started at 1500; the subjects went to bed and remained recumbent in total darkness until 2300, with polygraphic recording of sleep. At 2300, lights were switched on and the subjects had to get up and remain awake until the end of the experiment. Recumbency was not allowed during the scheduled waking periods. Breakfast and lunch were served at 0000 and 0430, respectively.

This schedule was enforced in both the placebo and the zolpidem studies. In the placebo study, the subjects were given a single oral dose of placebo at 1445 (i.e., 15 min before the 2 shifted bedtime periods), and light intensity was maintained around 200 lux during all ambulatory periods. In the zolpidem study, the subjects were given a single oral dose of 10 mg of zolpidem, a widely used nonbenzodiazepine hypnotic, at 1445 (i.e., 15 min before the 2 shifted bedtime periods), and dim-light conditions (±200 lux) were maintained during ambulatory periods.

Sleep Analysis

Polygraphic sleep recordings were scored at 20-s intervals in stages wake, I, II, III, IV, and rapid eye movement (REM) using standardized criteria (18). Sleep onset and morning awakening were defined, respectively, as the time of the first and last 20-s intervals scored as II, III, IV, or REM. The sleep period was defined as the time interval separating sleep onset from final morning awakening. Sleep efficiency was calculated as the total recording time minus the time spent awake, expressed as a percentage of the total recording time.

Hormonal Assays

Each blood sample was immediately centrifuged at 4°C. Plasma samples were frozen at −20°C until assay. Plasma melatonin levels were measured on dichloromethane-extracted plasma according to a procedure developed in our laboratory using an antimalatonin antiserum raised in a rabbit by Dr. J. P. Ravault (Nouzilly, France) and reagents supplied by Stockgrand (Dept. of Biology, Univ. of Surrey), with a lower limit of sensitivity of 2 pg/ml, an average intra-assay coefficient of variation of 10%, and an average interassay coefficient of variation of 15% in the range of physiological concentrations (22). All melatonin values that were at or below the level of assay sensitivity were considered equal to 2 pg/ml.

Analysis of Melatonin Profiles

The waveshape of each individual profile of plasma melatonin was quantified by a best-fit curve obtained using a robust locally weighted regression procedure proposed by Cleveland (7), with a regression window of 4 h. The acrophase and nadir were defined, respectively, as the time of occurrence of maxima and minima in the best-fit curve. The amplitude of the best-fit curve was defined as 50% of the difference between the value at the highest acrophase and the value at the lowest nadir.

For each subject and each study, baseline daytime melatonin levels were estimated as the mean levels measured during the first 4 h of the study (1100–1500). The melatonin onset was defined as the timing of the first plasma level exceeding the mean + 3 SD of baseline daytime levels not followed by a return to lower concentrations before the acrophase. The melatonin offset was defined as the timing of the last value occurring after the acrophase that exceeded the mean + 3 SD of baseline daytime levels. The amount of melatonin secreted was estimated by calculating the area under the curve between the onset and the offset.

Tests of Significance

Differences between study conditions and between consecutive days of investigation were evaluated by two-way ANOVA for repeated measures. Statistical calculations were performed using the StatView SE+ and SuperAnova (Abacus Concepts, Berkeley, CA) softwares for Macintosh computers. Unless otherwise indicated, all group values were summarized as means ± SE.

RESULTS

Melatonin

Figure 2 shows the mean plasma melatonin profiles observed during the two studies. The individual profiles of a representative subject are illustrated in Fig. 3. During the baseline period, the classic 24-h profile of plasma melatonin levels was observed in each study, with stable low daytime values until the onset of the circadian rise, which occurred around 2200 with remarkable reproducibility both within and across subjects. The acrophase was reached around midsleep. On average, melatonin levels returned to low daytime values between 0730 and 0800. Areas under the curve during the baseline period were similar for the two study conditions. In 5 of the 16 baseline profiles, a small and transient elevation of plasma melatonin levels was observed in the afternoon of the second day. This modest increase started between 1320 and 1540, lasted on average 176 ± 50 min, and did not appear related to any identifiable event (e.g., postural change, food intake, modification of light intensity).

Placebo study. The upper panel of Fig. 4 shows the mean profiles observed during the two shifted periods compared with baseline. The mean timings of the onset and offset of melatonin secretion are reported in Fig. 5. During the first postshift period, the melatonin onset was advanced in all subjects compared with baseline conditions, with the shift averaging 1 h 48 ± 11 min or 108 ± 11 min (P < 0.0001). Thus the melatonin onset occurred during the scheduled sleep period after ~5 h of exposure to darkness. At the end of the shifted bedtime period, i.e., after the lights were switched on at 2300, a partial inhibition of melatonin secretion was observed in five of eight subjects. At the group level, the decrease from the concentration observed immediately before lights-on (2300) was significant and, over the subsequent 2-h period, averaged 33 ± 10% (P < 0.01). The melatonin offset occurred ~6 h after lights-on and was advanced by 0 h 48 ± 18 min compared with baseline conditions (P = 0.11), i.e., 1 h less than the melatonin onset. The amplitude of the melatonin elevation and the area under the curve were similar to those recorded before the shift.

During the second shifted sleep period, there was a trend toward further adaptation of the melatonin pro-
files, but these additional advances failed to reach significance for either the onset or the offset (Figs. 4A and 5A). Compared with baseline, the shift of the onset of the melatonin elevation averaged 2 h 15 ± 18 min or 135 ± 18 min (P < 0.0001), and the shift of the offset averaged 1 h 13 ± 13 min or 73 ± 13 min (P < 0.02). Thus, as during postshift period 1, the onset shifted more than the offset. A significant drop in melatonin concentrations was observed in all subjects after lights-on, with a mean decrease from 2300 levels, averaging 43 ± 6% within the next 2 h (P < 0.0002). There were no modifications of amplitude or area under the curve.

Thus acute exposure to an 8-h advance of the sleep/dark period without pharmacological stimulation of sleep was associated with an immediate advance of the melatonin profiles.

Zolpidem study. The mean melatonin profiles during the study with zolpidem treatment are shown in Figs. 2B and 4B. Throughout the study, these profiles were entirely similar to those observed under placebo conditions. As illustrated in Fig. 5, under zolpidem, the phase advances of the melatonin onset and of the melatonin offset were similar to those observed under placebo, both on the first and on the second shifted periods.

Sleep

During both baseline nights, sleep parameters were normal for laboratory conditions.

Placebo study. When lights were turned off at 1500 for the first shifted sleep period, all subjects were able to fall asleep within 18 ± 4 min, but sleep efficiency was markedly reduced (P < 0.001), averaging only 50 ± 7%, and the amounts of stages I + II (P < 0.05), the slow-wave (SW) stage (P < 0.05), and the REM stage (P < 0.001) were all decreased, whereas the amount of wake was increased (P < 0.01). Two subjects woke up after only 342 min and 226 min of sleep, respectively, and were unable to reinitiate sleep. All other subjects experienced, around the middle of the dark period, an episode of sustained wakefulness lasting on average 157 ± 26 min. Sleep efficiency during the second shifted night was higher than during the first shifted night, averaging 71 ± 5% (P < 0.05), reflecting increases in
SW (P < 0.001) and REM (P < 0.05) stages and trends toward greater amounts of stages I+II.

Zolpidem study. The first dose of zolpidem was administered 15 min before the start of the first shifted bedtime period. This treatment did not affect the sleep-period time but increased sleep efficiency (64 ± 5 vs. 50 ± 7% in the placebo study, P < 0.05), primarily by eliminating the prolonged awakenings and increasing the amount of the SW stage (66 ± 6 vs. 42 ± 6 min in the placebo study, P < 0.02). However, the amount of REM was comparable to that observed in the placebo experiment, i.e., markedly reduced compared with baseline conditions. During the second shifted night, there were no differences in sleep parameters between the zolpidem study and the placebo study.

Analysis of Relationships Between Melatonin and Sleep

To examine a possible facilitation of sleep by elevated melatonin levels, the mean amount of wake was calculated in each 20-min interval between successive blood samplings during the two shifted nights under each study condition. There was no relationship between the timing of the melatonin onset and the timing of the reinitiation of sleep after prolonged awakenings interrupting sleep (r = 0.08, P > 0.70). A similar result was obtained when those nights under zolpidem treatment were excluded (r = 0.21, P > 0.50).

The quality of sleep during the shifted bedtime periods did not seem to have contributed to the advances in melatonin onset, since there were no correlations between the timing of the melatonin onset and the amounts of wake, stages I+II, SW stages, and REM stage. Similar results were obtained when only the first 4 h of the scheduled sleep period were considered as well as when the dependent variable was the magnitude of the shift from baseline conditions rather than the clock time of the melatonin onset.

DISCUSSION

In the present study, the analysis of the profiles of plasma melatonin during the first 40 h after an abrupt advance of the sleep–wake, dark–light, and feeding cycles revealed an unexpected rapid effect of exposure to dark during the afternoon and evening on markers of circadian phase. Indeed, a nearly 2-h advance of the melatonin onset appeared within 6 h after lights-out during the first shifted night. This phase advance was not limited to the onset of nocturnal secretion but also affected the offset. No significant additional phase advances were observed on the second day after the shift, indicating that exposure to dim light starting at the usual bedtime and extending for the next 16 h followed by exposure to a second advanced dark period was ineffective in promoting further adaptation to the shift. Bedtime administration of a hypnotic failed to facilitate adaptation of the melatonin profile to the advance shift.

At least two distinct mechanisms may underlie the unexpected observation of a rapid advance of the melatonin onset after 4–6 h of scheduled sleep in total darkness starting in the midafternoon. First, it could be hypothesized that under baseline conditions, evening dim light (± 200 lux) exerted masking effects on the melatonin onset, and that these masking effects were eliminated by prolonged exposure to darkness. However, recent studies that have measured the melatonin onset in a similar population of young, healthy subjects but under various conditions of illumination ranging from <10 to <300 lux, have consistently reported onset times around 2200–2300 (10, 14, 21, 22, 24), i.e., similar to those observed under baseline conditions in the present study (Table 1). Thus it appears likely that even if light intensities around 10 lux had been used under baseline conditions, similar phase advances of the melatonin onset would have been observed during dark exposure. The fact that the entire melatonin profile was advanced after dark exposure also argues against a simple unmasking mechanism. Indeed, in both studies, the advance of the melatonin offset occurred more than 3 h after the partial inhibition by light had been fully expressed and therefore is more likely to reflect a phase advance of the circadian control of melatonin secretion than a masking effect of dim light.

If the overall advance of the melatonin profile after afternoon dark exposure cannot be satisfactorily explained by masking/unmasking mechanisms, an alternative mechanism is that exposure to dark directly affects human circadian phase. Phase-response curves to dark pulses have been described in a number of nocturnal rodent studies (6, 11), but it appears that these phase-shifting effects are related to increased locomotor activity elicited by dark exposure, since they can be blocked by restraining the animals (19, 23). However, a phase-response curve to dark pulses has recently been described in a diurnal rodent, Octodon degus, and was shown to occur independently of changes in rest activity (15). The present study provides evidence that exposure to dark may have phase-shifting effects in another diurnal species. A role for the associated change in rest activity state cannot be excluded but is not supported by the lack of correlation between any measure of sleep quantity or quality and magnitude of the phase shift or by the observation that pharmacological facilitation of sleep was not associated with larger phase shifts than placebo treatment.

Table 1. Review of recent studies estimating timing of onset of melatonin secretion under different dim-light intensities

<table>
<thead>
<tr>
<th>Reference</th>
<th>Light Intensity, lux</th>
<th>Timing of Melatonin Onset, mean time (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>&lt;300</td>
<td>2219 (±50 min)</td>
</tr>
<tr>
<td>Present study</td>
<td>±200</td>
<td>2158 (±13 min)</td>
</tr>
<tr>
<td>24</td>
<td>&lt;100</td>
<td>2300</td>
</tr>
<tr>
<td>14</td>
<td>&lt;100</td>
<td>2300 (±96 min)</td>
</tr>
<tr>
<td>10</td>
<td>&lt;50</td>
<td>2200</td>
</tr>
<tr>
<td>21</td>
<td>&lt;50</td>
<td>2240</td>
</tr>
<tr>
<td>14</td>
<td>&lt;10</td>
<td>2224 (±78 min)</td>
</tr>
</tbody>
</table>
After the first advanced dark period, subsequent exposure to 16 h of dim light and a second advanced 8-h dark period failed to produce additional significant shifts of melatonin onset and offset. It is generally thought that the human circadian system is not sensitive to light intensities as low as 200 lux, although a recent report has suggested otherwise (5). If the extended period of exposure to dim light had an impact on circadian phase, it could have been in the delaying direction, because the 16-h advanced waking period encompassed both the phase-delay and the phase-advance regions of the human phase-response curve to light. In addition, the delay region is longer and larger than the advance region (9, 17, 22). The subsequent exposure to dark may have counteracted a phase delay induced by dim light. Alternatively, it is possible that this second advanced dark period no longer impacted the phase-advance region of the putative phase-response curve to dark.

In the present study, the observation of the melatonin onset on 3 consecutive days indicated that the overall phase shift was primarily achieved during the initial exposure to the 8-h period of dark. Interestingly, the “3-pulse” protocol, which has been used during the past decade by Czeisler and collaborators (5, 9) to derive the properties of the human phase-response curve to light of variable intensity, includes, as the first manipulation of photic environment, exposure to an 8-h period of sleep in darkness, scheduled at nearly the same clock time as the first shifted sleep period in our study. In these studies, circadian phase was generally measured before and after the 3-day protocol but was not tracked during the procedure. The finding in the present study of a rapid phase advance of almost 2 h in response to dark exposure suggests that some of the overall phase shifts interpreted as being the result of light exposure in the 3-pulse protocol could have, in fact, been primarily caused by dark exposure. In particular, the small advance shifts, averaging <1 h, observed in response to this 3-pulse protocol with dim light of ≥180-lux intensity (4, 5), may well have been caused by the initial exposure to dark.

Pharmacological facilitation of sleep with zolpidem, a widely used hypnotic drug, failed to facilitate adaptation of the melatonin profile. Beneficial effects on sleep quality were apparent on the first shifted night but were no longer detectable during the second shifted night. The analysis of the interrelationships between the sleep profiles and the melatonin profiles indicated that the elevations of endogenous melatonin levels during the shifted sleep periods were not associated with an improvement of sleep quality.

In conclusion, the present results indicate that afteroon exposure of young normal subjects to a sleep-dark period results within 6 h in an almost 2-h advance of melatonin onset and suggest that human circadian phase is sensitive to dark exposure.

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