J. Pineal Res. 2005; 39:195–200
Doi:10.1111/j.1600-079X.2005.00236.x

Journal of Pineal Research

Circadian phase determined from melatonin profiles is reproducible after 1 wk in subjects who sleep later on weekends

Abstract: The aim of this study was to determine whether circadian phase from salivary melatonin profiles is the same when measured in phase assessments 1 wk apart. Eleven healthy young men and women maintained a fixed, home sleep–wake schedule, in bed, in the dark 23:00–07:00 hr on weekdays. On Friday and Saturday nights they were permitted to wake up and go to bed up to 1 hr later, and on Saturdays and Sundays they could nap between 13:30 and 16:30 hr. The study was run in the summer. Subjects wore sunglasses when outside during the day, and went outside for at least 15 min between 08:00 and 09:00 hr each morning. They maintained this schedule for 15 days before the first assessment and the 6 days in between the two assessments. During the assessments subjects remained awake overnight in <5 lux and gave saliva samples every 30 min. A recovery nap (13:00–17:00 hr) followed the first session. The dim light melatonin onset (DLMO), offset (DLMOff) and midpoint were used as phase markers. There was minimal change in their timing between the two phase assessments. The average absolute change in midpoint (the change in phase regardless of direction) was 20 min. There was a small, 30 min delay in the DLMO. Thus, circadian phase can be measured a week in advance of any phase shifting intervention and, as long as the prescribed sleep and morning light schedule is maintained, the phase at the start of treatment can be confidently estimated.

Victoria L. Revell[1], Hyungsoo Kim[1], Christine Y. Tseng[1], Stephanie J. Crowley[2] and Charmane I. Eastman[1]

[1]Biological Rhythms Research Lab, Rush University Medical Center, Chicago, IL; [2]Department of Psychology, E.P. Bradley Hospital Sleep and Chronobiology Research Laboratory, Brown University, Providence, RI, USA

Key words: melatonin, morning light, sleep

Address reprint requests to Dr Victoria Revell, Biological Rhythms Research Lab, Rush University Medical Center, 1645 W Jackson Blvd, Suite 425, Chicago, IL 60612, USA.
E-mail: victoria_l_revell@rush.edu

Received December 21, 2004;
accepted March 21, 2005.

Introduction

In humans, the circadian rhythm of melatonin is often used as a marker for the phase of the circadian clock [1], but melatonin must be sampled in dim light because light suppresses melatonin secretion [2]. A number of different points on the melatonin profile are used as phase markers of the clock, including the dim light melatonin onset (DLMO), the dim light melatonin offset (DLMOff) and the midpoint between the DLMO and DLMOff.

Phase shifting studies, such as simulated and real night work and jet lag studies frequently involve sleep deprivation because the timing of sleep is shifted to daytime hours following night shifts or to night time in the new time zone. Bright light, dark and exogenous melatonin are often used to phase shift the clock, but they must be applied at appropriate times to produce the desired shift. Ideally, baseline phase should be determined immediately before any phase-shifting stimuli are applied, but if the phase assessment session involves subjects remaining awake overnight then this will result in the subjects being in a sleep-deprived state when the treatment begins. In addition to obvious burdens to the subjects, it is also possible that sleep deprivation itself could attenuate the magnitude of the phase shift as shown in rodents [e.g. 3]. A further disadvantage of measuring the baseline phase position on the day before treatment is that assays for melatonin take a few days so the baseline phase position can only be calculated retrospectively and thus the treatment cannot be precisely timed.

Two strategies would allow baseline phase to be determined from saliva samples whilst ensuring that the subjects begin treatment in a non-sleep deprived state. A shortened phase assessment, with samples only being taken in the early evening to determine the timing of the DLMO, would not require the subjects to stay awake overnight [e.g. 4]. The major disadvantage of this design is that if the whole melatonin profile is not collected then the DLMOff and midpoint cannot be determined. An alternative option is to carry out the baseline phase assessment several days before treatment and allow subjects to recover from any sleep deprivation. In addition, this would allow time for the samples to be assayed for melatonin and for baseline phase to be calculated. This method relies on the assumption that circadian phase does not change in between the baseline phase assessment and the first day of treatment, or if it does that it returns to baseline before the treatment begins.

To test this alternative, we carried out two circadian phase assessments 1 wk apart. Subjects followed a prescribed sleep and outdoor morning light schedule before and in between the two sessions which were both on Tuesdays. Subjects were allowed to sleep 1 hr later and nap on weekends. We show that given these conditions circadian phase was very similar in the two sessions.

Materials and methods

Twelve subjects completed the study, but one was excluded from data analyses because of low melatonin levels. The remaining eleven subjects (five men and six women) were between 19 and 39 yr old (mean ± S.D., 26.6 ± 5.9 yr). Subjects had no medical, psychological, or sleep disorders as assessed by a self-assessed health and medical history questionnaire, the Minnesota Multiphasic Personality Inventory-2 and the Pittsburgh Sleep Quality Index [5]. Subjects were not taking prescription medications and had body mass indexes ≤ 30. Urinary toxicological analysis was performed to verify that they were free from common recreational drugs at the start of the study. None were definite (extreme) morning or evening types, as assessed by the Morningness-Eveningness Questionnaire [6]. No subjects had worked night shifts in the 3 months prior to the start of the study or traveled across more than three time zones in the month prior to the start of the study. The study protocol was approved by the Rush University Medical Center Institutional Review Board. All subjects gave written informed consent and received payment for their participation.

Protocol

For 15 baseline days subjects slept at home and had to go to bed at 23:00 hr and wake up at 07:00 hr on weekdays. On Friday and Saturday nights they were allowed go to bed between 23:00 and 00:00 hr and wake up between 07:00 and 08:00 hr on the following morning. See Fig. 1 which will be described in more detail in the Results section. Subjects were required to remain in bed in the dark during the required times, except for necessary bathroom trips, even if they were awake. Naps were allowed on the weekends between 13:30 and 16:30 hr, i.e. centered 12 hr away from the midpoint of weekday sleep. Every morning, on both weekdays and weekends, subjects were required to go outside to receive natural light for a minimum of 15 min between 08:00 and 09:00 hr. Throughout the study, subjects wore blue-blocker sunglasses (UVEX Bandido frame, espresso lens, 0 to about 25% visual light transmission depending on wavelength) when going out during the daytime. On day 16 (Tuesday), subjects came to the lab in the afternoon for the first circadian phase assessment. They left the lab around noon on Wednesday and were required to take a nap between 13:00 and 17:00 hr. Then they resumed the baseline sleep schedule until day 23 (Tuesday) when they returned for the final phase assessment. Subjects were allowed to consume up to 300 mg of caffeine per day before 17:00 hr and up to two alcoholic drinks per night, except on the days before the phase assessments. Subjects participated in August, September and October, and all times are reported in daylight savings time.

Measures of sleep

Subjects completed a daily sleep log for each day of the study. Right before their bedroom lights were turned off they recorded their bedtime. Immediately after awakening they filled in their wake time, estimated sleep onset and any night time wake episodes with durations longer than 5 min. They recorded any naps on separate sleep logs. Every day, subjects were required to call the laboratory's time-stamp voice mail just before bedtime and immediately upon waking. Throughout the study the subjects were required to wear an actigraphy monitor (Actiwatch-L, MiniMitter Inc., Bend, OR, USA) on their nondominant wrist. The subjects were required to visit the laboratory every 2–3 days to verify their recorded bed and wake times on the daily sleep logs with the actigraphy data.

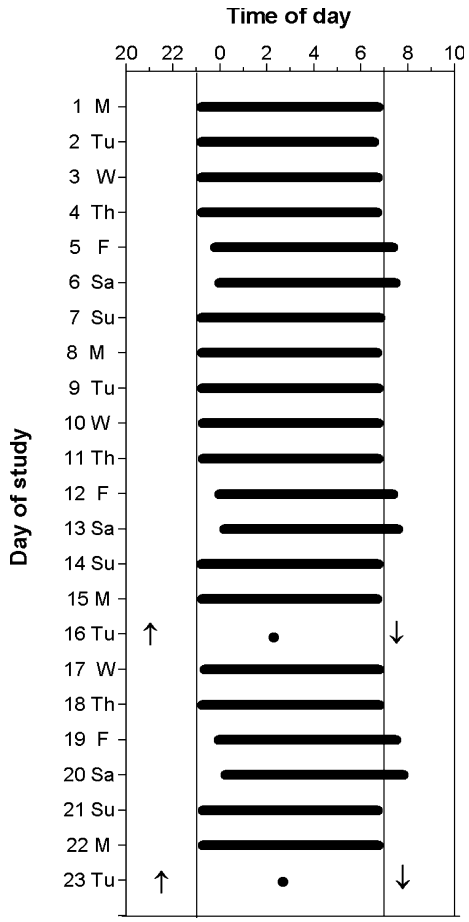

Fig. 1. Average time in the dark, from bedtime to wake time, from sleep logs. Vertical lines indicate scheduled weekday bedtime (23:00 hr) and wake time (07:00 hr). Subjects were allowed to go to bed as late as midnight and wake up as late as 08:00 hr on weekends. Average DLMO, midpoint and DLMOff during each phase assessment are depicted as up arrows, filled circles and down arrows respectively.

Measures of light exposure

Light exposure was recorded throughout the study using a photosensor worn as a medallion around the subjects' neck (Actiwatch-L, MiniMitter Inc.). The photosensor at this position captures light intensity at eye level more closely than when worn on the wrist. Subjects wore the photosensor at all times except during sleep, showers and baths. During sleep subjects put the photosensor face up on a nightstand in their bedroom. Every 2–3 days, the photosensor data were

Table 1. Sleep parameters averaged for the 6 days before the first phase assessment (days 10–15) and for the 6 days before the second phase assessment (days 17–22)

Type of night	Phase assessment	Bedtime (S.D. in min)	Sleep onset (S.D. in min)	Wake time (S.D. in min)	Sleep duration (S.D. in min)
Weekdays	First	23:02 ± 3	23:26 ± 12	6:56 ± 8	7.5 ± 0.3
	Second	23:02 ± 5	23:21 ± 13	6:58 ± 3	7.6 ± 0.2
Weekends	First	23:52 ± 13	00:12 ± 21	7:41 ± 17	7.5 ± 0.3
	Second	23:52 ± 13	00:08 ± 17	7:52 ± 16	7.7 ± 0.4

checked in the subjects' presence to verify their minimum 15-min exposure to natural outdoor light between 08:00 and 09:00 hr.

Assessment of circadian phase

Saliva samples were collected using Salivettes (Sarstedt, Newton, NC, USA) at 30-min intervals, starting at 15:30 hr on Tuesday and ending at 12:00 hr on Wednesday. The sampling ended at 11:00 hr in the second assessment for the last four subjects. The samples were centrifuged immediately after collection and stored in a freezer. The melatonin levels were measured by radioimmunoassay analysis, performed by Pharmasan Labs (Osceola, WI, USA). All samples from a single subject were assayed together. The intra-assay and inter-assay variability were 12.1 and 13.2% respectively; the sensitivity of the assay was 0.7 pg/mL.

During the phase assessments subjects remained awake, sitting in recliners in < 5 lux. They were only allowed to leave the recliner to go to the washroom (also maintained at < 5 lux) but otherwise their physical activities were kept minimal. Subjects could eat and drink during the assessment but if they did so they had to brush their teeth and rinse with water (whilst remaining seated) 10 min prior to giving a saliva sample. For the 10 min before each saliva sample subjects had to remain seated and were not allowed to eat or drink anything. On the days of and throughout the phase assessment sessions caffeine, chocolate, bananas, and lipstick were not allowed. Toothpaste and mouthwash were not permitted during the assessment sessions. Alcohol was prohibited in the 24 hr preceding the assessments and subjects were breathalyzed at the beginning of the assessment to verify their compliance. Nonsteroidal anti-inflammatory drugs were only allowed on days 1–11 of the study.

Data analysis

Light

Data from the light sensor worn around the neck were analyzed for the days in between the two phase assessments. We assumed that the subject was outside when light levels were > 500 lux and then we corrected for the transmission of the sunglasses (12%). Then the light levels were averaged into 1-hr bins.

Melatonin phase markers

The melatonin profiles were smoothed with GraphPad Prism, which employs locally weighted regression scatter plot smoothing (LOWESS) [7, 8]. We used the high level of smoothing. The DLMO was defined as the first minute of the smoothed curve which exceeded and stayed above a threshold value. The DLMOff was defined as the point where the profile went below, and remained under, the threshold. The threshold was determined from the raw data as the average of the five lowest continuous data points at the beginning of the profile plus 15% of the average of the five highest continuous data points. This method was used, instead of a fixed absolute threshold or one based on only the low daytime values, as it takes into account both the basal daytime levels and the amplitude of the rhythm which can vary widely between individuals. This threshold occurs low on the profile as melatonin levels are just starting to rise and thus the calculated DLMO is close to when the pineal gland starts to secrete melatonin. We believe this is preferable to thresholds that occur at a higher point on the profile, such as 50% amplitude, because these will be more influenced by individual differences in melatonin metabolism. We calculated each subject's phase change from the first to the second phase assessment and the average for all three markers. However, it is possible that some subjects could advance while others delay canceling each other out such that it would appear that there had been

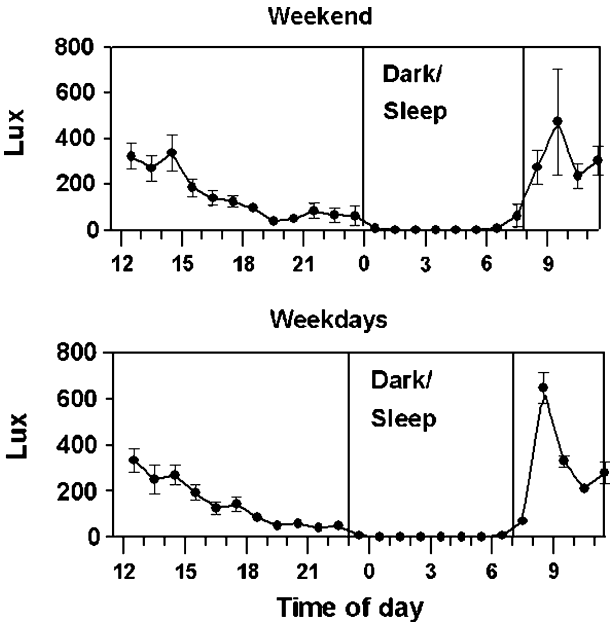

Fig. 2. Average light exposure during the 6 days between phase assessments. The light data were averaged into 1-hr bins. Light intensity level was corrected for sunglasses (12% transmission). Two vertical lines enclose average bed and wake times for weekdays and for weekend. Error bars represent S.E.M.

no change in phase. Therefore we also calculated absolute phase changes that do not take into account the sign of the phase change to better determine the reproducibility of our phase markers.

Sleep analysis

Sleep logs were used to calculate the bedtime, estimated sleep onset and final wake time. Sleep duration was the time between sleep onset and final wake time. We also calculated the intervals between the phase markers from each phase assessment session and the average sleep parameters (bedtime, estimated sleep onset, wake time) from the preceding 6 days for both weekdays and weekends.

Statistical Analysis Paired *t*-tests were used to compare the 3 phase markers between the first and second phase assessments and similarly to compare 3 of the intervals between phase markers and sleep. To account for the fact that 3 separate *t*-tests were used in each case, a Bonferroni correction was applied and a significance level of $p < 0.01$ was used.

Results

Fig. 1 and Table 1 show sleep times. Almost all subjects went to bed and woke up as late as permitted on the weekends. There were no significant differences in any of the sleep parameters between the 6 days preceding the first phase assessment and the 6 days preceding the second phase assessment (Table 1). Very few subjects took the permitted weekend naps; five subjects took naps in the weekend before the first phase assessment and three subjects in the weekend before the second phase assessment session. The average nap duration was 43 min and it was the same on both weekends.

Fig. 2 shows the light exposure pattern, corrected for sunglasses worn when outside. Recall that subjects went outside for at least 15 min between 08:00 and 09:00 hr, accounting for the high values at 8:30 hr (the average from 08:00 to 08:59 hr). The value of about 300 lux at 08:30 hr on the weekends corresponds to about 2500 lux before the light is attenuated by sunglasses. Thus, the first relatively bright light after waking (compared with the dark during sleep) occurred at 08:30 hr on both weekends and weekdays.

The individual profiles of all 11 subjects had characteristic shapes that were similar in both phase assessments (Fig. 3). Some people had steep rising phases and more gradual falling phases (e.g. S8), some had gradual rising phases and steep falling phases (e.g. S10) and others assumed other characteristic shapes. There were also large differences among subjects in the levels of melatonin

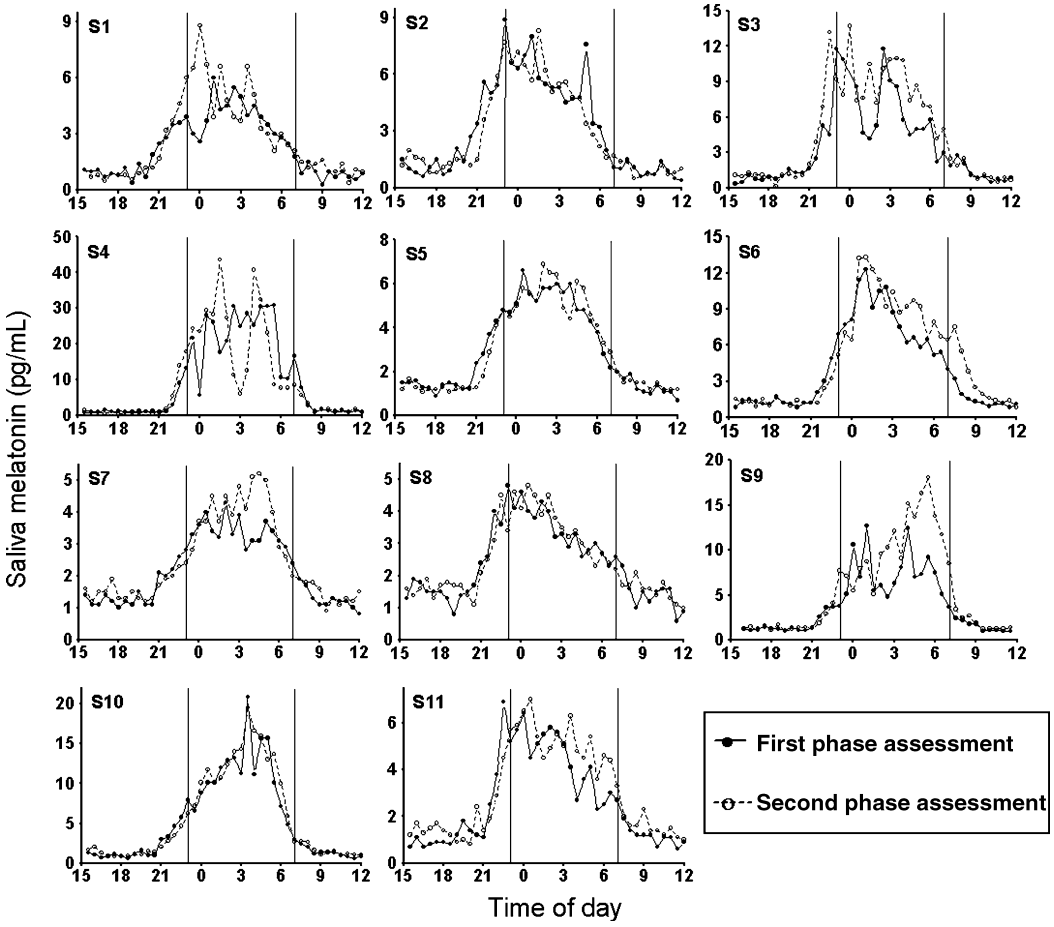

Fig. 3. Raw salivary melatonin profiles from all subjects. The two vertical lines represent scheduled, weekday bedtime (23:00 hr) and wake time (07:00 hr).

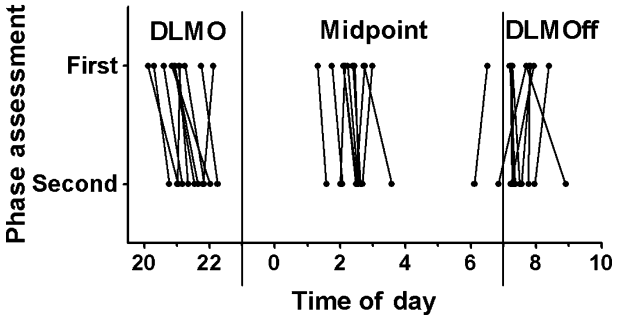

Fig. 4. Phase markers of individual subjects during the first and second phase assessments. Phase markers from each phase assessment are connected for each individual. The two vertical lines represent scheduled weekday bed and wake times.

detected (e.g. compare S4 and S8). The DLMO threshold ranged from 1.30 to 5.33 pg/mL and the mean was 2.46 ± 1.05 pg/mL (mean ± S.D.).

As shown in Fig. 4 there were small phase changes in all phase markers for most subjects. Table 2 shows that the time of the phase markers was similar during both phase assessments. However, there was a significant phase delay of 30 min in the timing of the DLMO between the two phase assessments ($t = -3.93$, d.f. = 10, $P = 0.003$). There were small, non-significant phase shifts in the midpoint and DLMOff. The mean absolute phase changes were also small ranging from 20 to 34 min. Table 3 shows that the interval between the DLMO and sleep onset was about 2–3 hr and that the interval between wake time and DLMO was about 13–14 hr. As the DLMO delayed slightly while sleep times remained the same, the interval between the DLMO and sleep onset was slightly reduced, and the interval between wake and the DLMO was slightly increased, for the second phase assessment. The time of the DLMOff did not change between phase assessments, but it occurred slightly after wake time on weekdays and slightly before wake time on weekends.

Discussion

We found that the times of the melatonin circadian phase markers were very similar in phase assessments 1 wk apart. There was a small but significant 30 min delay in the DLMO, essentially no change in the DLMOff and a very small non-significant delay in the midpoint. However, this does not mean that the DLMOff is the most stable marker. The absolute phase changes, the magnitude of the phase change regardless of direction and thus the best indicator of reproducibility, were slightly smaller for the midpoint than the DLMO or DLMOff. On average the midpoint changed by 20 min between phase assessments. However, we think that any of the phase markers could be used to predict circadian phase 1 wk after a phase assessment procedure like ours, as long as subjects follow the same sleep and morning outdoor schedule both before and after the baseline phase assessment. This means that in future studies baseline assessments could be conducted 1 wk before any phase shifting intervention was applied, so that the subjects could recover from the sleep loss before beginning a protocol that involves further sleep deprivation.

It is interesting to consider what caused the small phase delay in the DLMO. Possibilities include the sleep deprivation of the phase assessment, the dim light (< 5 lux) during the phase assessment when subjects would otherwise be asleep in the dark, and the recovery nap from 13:00 to 17:00 hr after the phase assessment. We doubt that the 4 hr recovery nap which started about 16 hr after the baseline DLMO could have much of a phase delaying effect, because Buxton et al. [9] showed that a 6-hr afternoon nap starting about 15 hr after the DLMO did not change circadian phase. This leaves the dim light and sleep deprivation of the phase assessment as the most likely factors for the delay. In any case, the DLMO was more affected than the DLMOff, suggesting a larger or more reliable effect on the putative evening oscillator than the morning oscillator. It is also conceivable that both phase markers delayed and then

Table 2. Circadian phase positions and phase changes in the melatonin phase markers

Phase marker	First phase assessment (mean clock time ± S.D., min)	Second phase assessment (mean clock time ± S.D., min)	Phase shift[a] (mean ± S.D., min)	Absolute phase change[b] (mean ± S.D., min)
DLMO	21:00 ± 35	21:30 ± 28*	−30 ± 25	34 ± 18
Midpoint	2:15 ± 28	2:28 ± 30	−13 ± 20	20 ± 12
DLMOff	7:32 ± 30	7:27 ± 41	5 ± 34	24 ± 23

[a]Negative numbers indicate a phase delay, positive numbers indicate a phase advance.
[b]All phase changes were converted to positive values before averaging.
*$P < 0.01$ compared with the first phase assessment with a paired t-test.

Table 3. Intervals between sleep parameters from the 6 days before each phase assessment and the melatonin phase markers

Interval	Type of night	First phase assessment (mean ± S.D., hr)	Second phase assessment (mean ± S.D., hr)
DLMO to sleep onset	Weekday	2.4 ± 0.6	1.9 ± 0.5*
	Weekend	3.2 ± 0.5	2.6 ± 0.5*
Wake to DLMO	Weekday	14.1 ± 0.6	14.5 ± 0.5*
	Weekend	13.3 ± 0.5	13.6 ± 0.5
Wake to DLMOff	Weekday	0.6 ± 0.6	0.5 ± 0.7
	Weekend	−0.2 ± 0.5	−0.4 ± 0.7

*$P < 0.01$ compared with the first phase assessment with a paired t-test.

advanced in ensuing days due to the regulated sleep schedule and morning light exposure, but that the DLMO did not advance as much as the DLMOff. Our subjects could have been very sensitive to the dim light of the phase assessments because they wore blue-blocker sunglasses whenever they went outside. Exposure to dim light for 1 wk (because of minimal outdoor light exposure and wearing very dark goggles when outside) has previously been shown to increase photic sensitivity as assessed with melatonin suppression [10]. Thus, the dim light of the phase assessment coinciding with the phase delay portion of the phase–response curve could have been at least partly responsible for the small delay. It is also possible that the sleep deprivation itself produced a small phase delay [cf. 11].

There are a few other studies with phase assessments that involved sleep deprivation and were a few days apart. In one [12] the two assessments were 7 days apart, and the circadian rhythm of the urinary melatonin metabolite 6-sulphatoxymelatonin (aMT6s) was measured in eight men on a fairly regular sleep–wake schedule (45 min window for bedtime and wake up). There was a 40-min absolute change in acrophase, a phase delay of 45 min in the onset and a phase advance of 45 min in the offset. These changes are all larger than those in the current study, and could have been due to the longer phase assessments and concomitant sleep deprivation and dim light exposure. In another study [13] constant routines were conducted 4 days apart in subjects who were either free-running or delaying until a new stable phase relationship could be reached relative to their LD cycle, because they were kept in very dim light (5–13 lux) whenever awake. The delay in the circadian rhythm of plasma melatonin was greater on the day of and the day after the constant routines compared with the intervening days, and this was particularly noticeable in the melatonin offset. Again this could have been because of either the dim light exposure or sleep deprivation during the constant routines.

There have also been a few studies with multiple phase assessments that did not involve sleep deprivation. One of these studies [14] is only reported in abstract form and the magnitude of phase changes are not reported; only that the markers were significantly correlated over a 3-wk period in subjects instructed to maintain a regular sleep schedule in between phase assessments. In another study [15] there were three, 24-hr phase assessments over a 6-wk period. The authors concluded that melatonin profiles were highly reproducible, but the profiles of some individuals showed much greater variability than any of ours. This could be attributable to the range of sleep times allowed (23:00–08:00 $\pm$ 1 hr) before, and presumably in between, the sampling sessions or exposure to bright indoor light in between samples as subjects were free to leave the lab.

Future work is necessary to determine the minimum restrictions needed to obtain reproducible melatonin phase markers. We found that phase could be predicted fairly accurately 1 wk after an initial phase assessment in subjects on a fixed weekday sleep schedule who slept 1 hr later on the weekends, but always got morning light within the same 1 hr interval on both weekdays and weekends. In addition, individual melatonin profiles had a replicable, characteristic shape which also demonstrates the stability of the melatonin rhythm within subjects, and thus its reliability as a phase marker of the circadian clock.

Acknowledgments

We thank Clifford Gazda and Clara Lee for assistance in data collection and Dr Helen Burgess for helpful comments on the manuscript. Support for this study came from the National Institute of Occupational Health and Safety (NIOSH), Centers for Disease Control and Prevention (CDC) grant R01 OH003954. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of NIOSH or the CDC.

References

1. Lewy AJ, Cutler NL, Sack RL. The endogenous melatonin profile as a marker of circadian phase position. J Biol Rhythms 1999; **14**:227–236.
2. Lewy AJ, Wehr TA, Goodwin FK et al. Light suppresses melatonin secretion in humans. Science 1980; **210**:1267–1269.
3. Mistlberger RE, Landry GL, Marchant EG. Sleep deprivation can attenuate light-induced phase shifts of circadian rhythms in hamsters. Neurosci Lett 1997; **238**:5–8.
4. Burgess HJ, Crowley SJ, Gazda CJ et al. Preflight adjustment to eastward travel: 3 days of advancing sleep with and without morning bright light. J Biol Rhythms 2003; **18**:318–328.
5. Buysse DJ, Reynolds CF, Monk TH et al. The Pittsburgh sleep quality index: a new instrument for psychiatric practice and research. Psychiatry Res 1989; **28**:193–213.
6. Horne JA, Ostberg O. Self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. Int J Chronobiol 1976; **4**:97–110.
7. Cleveland WS. Robust locally weighted regression and smoothing scatterplots. J Am Stat Assoc 1979; **74**:829–837.
8. Chambers JM, Cleveland WS, Kleiner B et al. Graphical Methods for Data Analysis. Duxbury Press, Boston, MA, 1983.
9. Buxton OM, L'Hermite-Baleriaux M, Turek FW et al. Daytime naps in darkness phase shift the human circadian rhythms of melatonin and thyrotropin secretion. Am J Physiol Regul Integr Comp Physiol 2000; **278**:R373–R382.
10. Hebert M, Martin SK, Lee C et al. The effects of prior light history on the suppression of melatonin by light in humans. J Pineal Res 2002; **33**:198–203.
11. Antle MC, Mistlberger RE. Circadian clock resetting by sleep deprivation without exercise in the Syrian hamster. J Neurosci 2000; **20**:9326–9332.
12. Dawson D, Lushington K, Lack L et al. The variability in circadian phase and amplitude estimates derived from sequential constant routines. Chronobiol Int 1992; **9**:362–370.
13. Cajochen C, Jewett ME, Dijk DJ. Human circadian melatonin rhythm phase delay during a fixed sleep-wake schedule interspersed with nights of sleep deprivation. J Pineal Res 2003; **35**:149–157.
14. Benloucif S, Guico MJ, Reid KJ et al. Stability of circadian phase markers under regular sleep schedules. Sleep 2003; **26**:A106.
15. Selmaoui B, Touitou Y. Reproducibility of the circadian rhythms of serum cortisol and melatonin in healthy subjects: a study of three different 24-h cycles over six weeks. Life Sci 2003; **73**:3339–3349.