Is Sleep per se a Zeitgeber in Humans?

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Abstract It is not clear whether shifting of sleep per se, without a concomitant change in the light-dark cycle, can induce a phase shift of the human circadian pacemaker. Two 9-day protocols (crossover, counterbalanced order) were completed by 4 men and 6 women (20-34 years) after adherence to a 2330 to 0800 h sleep episode at home for 2 weeks. Following a modified baseline constant routine (CR) protocol on day 2, they remained under continuous near-darkness (< 0.2 lux, including sleep) for 6 days. Four isocaloric meals were equally distributed during scheduled wakefulness, and their timing was held constant. Subjects remained supine in bed from 2100 to 0800 h on all days; sleep was fixed from 2330 to 0800 h in the control condition and was gradually advanced 20 min per day during the sleep advance condition until a 2-h difference had been attained. On day 9, a 25 to 27 h CR protocol (~0.1 lux) was carried out. Phase markers were the evening decline time of the core body temperature (CBT) rhythm and salivary melatonin onset (3 pg/ml threshhold). In the fixed sleep condition, the phase drift over 7 days ranged from +1.62 to –2.56 h (for both CBT and melatonin rhythms, which drifted in parallel). The drifts were consistently advanced in the sleep advance schedule by +0.66 ± 0.23 (SEM) h for CBT ($p = 0.02$) and by 0.27 ± 0.14 h for melatonin rhythms ($p = 0.09$). However, this advance was small to medium according to effect size. Sleep per se may feed back onto the circadian pacemaker, but it appears to be a weak zeitgeber in humans.

Key words sleep, nonphotic zeitgeber, human circadian rhythms, temperature, melatonin

In the hierarchy of environmental stimuli entraining human circadian rhythms, the light-dark cycle is primary, and the relative strength of other zeitgebers still remains to be elucidated. Under dim light conditions, the circadian pacemaker free runs with an endogenous period, on average, slightly longer than the 24-h solar cycle (Czeisler et al., 1999). Since there is evidence that even a low-intensity light-dark cycle of 10 to 200 lux can counteract this phase drift, earlier studies on the zeitgeber role of sleep and other nonphotic stimuli have been confounded with parallel shifts in a too-bright environmental light-dark cycle (for a review, see Klerman, 2001).

The most controlled studies of sleep as a putative zeitgeber were carried out under < 15 lux light, during which the main sleep episode was displaced to earlier or later. These studies showed small phase shifts in melatonin and/or core body temperature (CBT) rhythms after a 2-h sleep advance or delay (Hoban et al., 1991), 3-h sleep advance or delay (Gordijn et al., 1999; Jelínková-Vondrašová et al., 1999), half-hour daily gradual advance (Nakamura, 1996), or 12-h

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sleep displacement (Zeitzer et al., 1997). Two recent studies, published after the present study had begun, also suggested that sleep is a weak zeitgeber in humans (Miyazaki et al., 2001; Wright et al., 2001). Although light intensity in these studies was appropriately dim, behavioral factors such as meals and posture were not controlled or were shifted in parallel with the timing of sleep.

The present experiment tried to keep as many factors as possible constant apart from sleep timing: near-darkness (< 0.2 lux during scheduled wake and sleep episodes), activity limited within the laboratory, 4 isocaloric meals at the same time each day, and lying supine in bed every night during the same clock time from 2100 to 0800 h. Sleep was slowly advanced 20 min each day, and constant routine (CR) protocols established circadian phase before and after 6 days under these controlled conditions. We hypothesized that advancing scheduled sleep by 20 min each day in near-darkness cannot advance human circadian rhythms and that the subjects will drift as expected under the control condition of a fixed sleep-wake cycle.

MATERIALS AND METHODS

Participants

The study was carried out in Novosibirsk (55 °N) between January and June 2001. Subjects were recruited via advertisements in a local newspaper. During the screening interview with the principal investigator, the volunteers read the consent form, the study approval of the Ethics Committee of the Institute of Physiology SB RAMS, and filled out a questionnaire about their general health and daily habits (including the Pittsburgh Sleep Quality Index Questionnaire). Qualified volunteers were invited to the laboratory to be introduced to the experimental conditions, to undergo a general medical examination, and to sign the consent form.

The study participants were in good medical and psychological health, were nonsmokers, had no self-reported sleep disorders, did not use medications or recreational drugs, and had not engaged in shift work or transmeridian travel 2 months before the study began. Their habitual bedtime was between 2200 and 0100 h and 0630 and 1030 h in the morning. The study comprised two 9-day protocols in the laboratory (counterbalanced order) separated by an off-protocol episode of 16 to 22 days outside the laboratory. Women were studied during the follicular phase of their menstrual cycle. Twelve subjects entered the study, and 10 (4 men and 6 women, mean age = 24.9 ± 1.4 years, age range = 20-34 years) completed both arms of the study. Two dropouts occurred at the end of the 1st session: one for lack of motivation and the other with a psychological stress reaction. The latter did not require any specific treatment. Study participants were admitted to the laboratory as a group of 2 or 3, men and women separately. The resultant number of people during experimental sessions was 3 (3 sessions), 2 (6 sessions), and 1 (1 session), respectively.

Experimental Protocol

The experimental protocol is depicted in Figure 1. Two weeks before each laboratory session, participants were asked to maintain a strict sleep schedule from 2330 h ± 10 min to 0800 h ± 30 min, documented by sleep diary and wrist actimetry (Gähwiler™, Zürich) for the past 9 days. Compliance was checked upon arrival in the laboratory at 2100 h on day 1. The laboratory was a 3-room apartment with a separate bedroom for each participant. On day 2, the subjects were adapted to < 30 lux until the start of the modified baseline CR protocol at 1500 h. The modified CR was identical to "classical" CR protocols (Czeisler et al., 1985) but differed in being shorter, and sleep was permitted from 2330 to 0800 h under 0 lux (for details, see Kräuchi et al., 1997).

From day 3 on, the participants remained under continuous < 0.2 lux light, also during scheduled sleep episodes (they were asked to keep their eyes closed even if not asleep). The very dim light level was obtained with twenty 2.6-watt bulbs (a garland with a light regulator) spread on the perimeter of the rooms, and it was sufficient for staff members to verify that participants' eyes remained open. The dim whitish light was well diffused, with less than 0.2 lux at a height of 2 m and ~0.1 lux at a height of 1 m. Since the light was too low to read by, subjects were kept awake by computer (< 0.15 lux) and other sedentary games, audio tapes, social interaction, and continuous attention from the technician. Visitors were allowed between 1200 and 1900 h if subjects were not on a CR protocol. Four isocaloric meals, provided by a technician from a kitchen outside the experimental apartment, were equally distributed during the scheduled wake episodes and not shifted. To avoid postural confounds, subjects remained in bed from 2100 to 0800 h

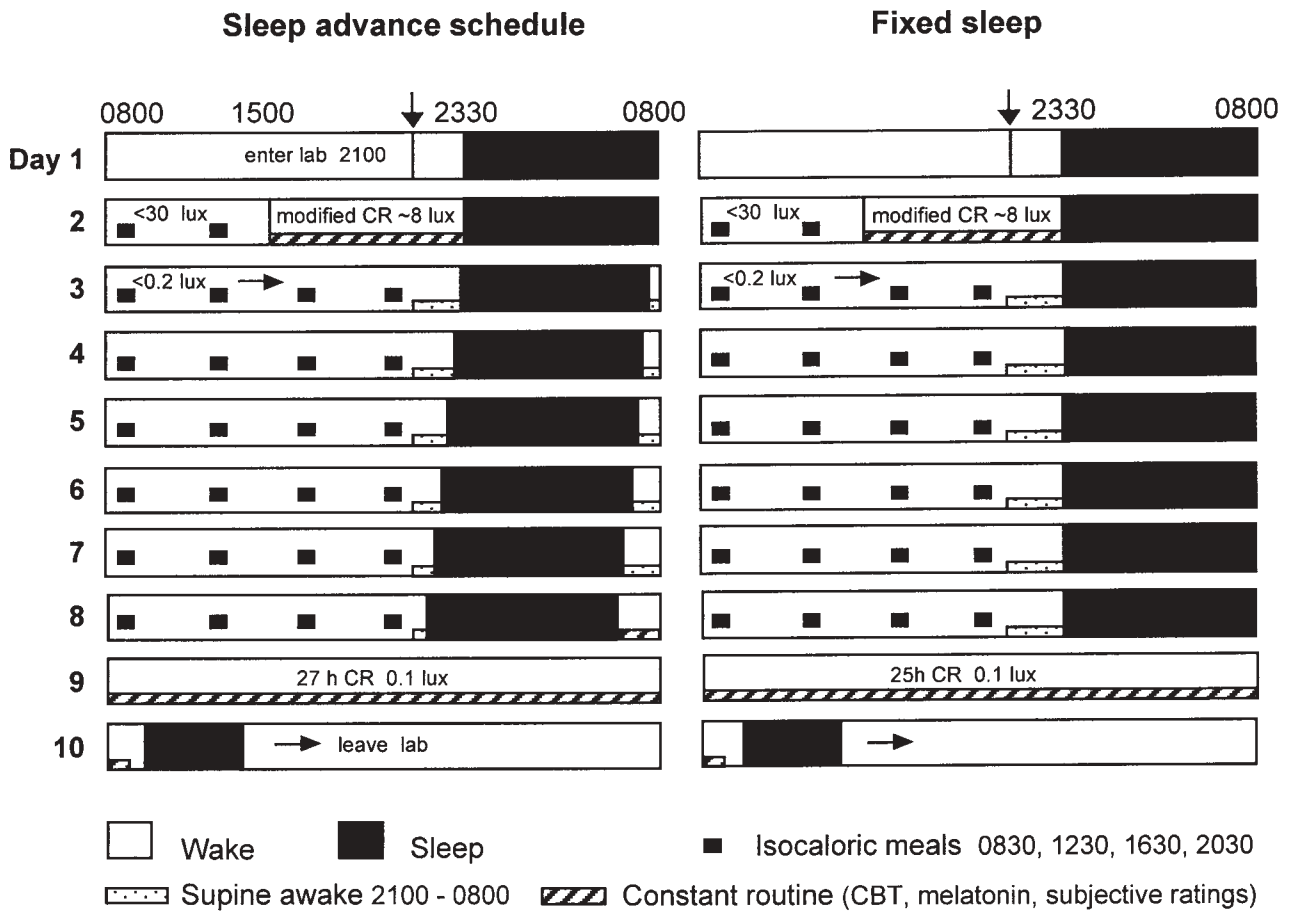

Figure 1. Schematic protocol of the 9-day sleep advance experiment. After habituation to laboratory conditions on day 1, the participants underwent an 8.5-h modified constant routine procedure on day 2 to measure the initial phase of core body temperature (CBT) and salivary melatonin circadian rhythms together with subjective ratings. Beginning from day 3, the subjects remained under continuous < 0.2 lux light level (including sleep periods). Sleep was advanced 20 min/day for 6 days during the experimental session (left panel) and remained stable during the control situation (right panel; crossover, counterbalanced order; interval between sessions = 16-22 days). A 25- to 27-h constant routine (CR) protocol was carried out at the end of the protocol, during which the final phase of CBT and melatonin circadian rhythms were measured.

on all days during both arms of the protocol. Sleep was permitted from 2330 to 0800 h in the control situation (i.e., fixed sleep). Sleep timing was gradually advanced 20 min/day during the experimental session (i.e., advanced sleep). Participants had access to clock time except during the CR protocols. On day 9, a 25- to 27-h CR protocol was carried out. Subjects remained in bed from 2100 h on day 8 until 0900 h on day 10, resulting in a total sleep deprivation during the night of days 9 and 10.

Measurements and Analyses

Subjects kept daily sleep logs, and actimetry data were collected at 2-min intervals throughout the entire study. The time of sleep onset and offset was determined by 2 independent raters. Absence of activity values ("0") during 5 consecutive 2-min epochs (10 min) was taken as the objective criterion. In both CR protocols, subjective estimates of sleepiness were assessed at 30-min intervals using the Karolinska Sleepiness Scale (Gillberg et al., 1994), together with 2 visual analog scales for energy and mood. CBT was recorded using a rectal probe and collected in 2-min intervals by a portable minilog device (ARM production, Novosibirsk; resolution = 0.026-0.031 °C). Saliva (2-4 ml), for the assessment of melatonin, was collected at 30-min intervals during the CR protocols, and an additional set of saliva samples was collected hourly in the evening of day 5 and the morning of day 6 as an intermediate phase marker. The samples were immediately refrigerated at 5 °C, centrifuged

within 2 days, and stored at –20 °C. Melatonin was assayed using highly specific, direct double-antibody radioimmunoassay kits from Bühlmann Laboratories (Weber et al., 1997).

As previously described (Kräuchi et al., 1997), the declining portion of the CBT curves was used to estimate phase markers. Briefly, the raw data were smoothed by 1-h moving averages, then the time of 1/3 range crossing between maximum and minimum (night 2 and night 8) of the temperature curve was defined as the temperature phase marker (CBT 1/3). The dim light melatonin onset time (DLMO), as determined by linear interpolation of the evening melatonin rise across a 3 pg/ml threshold, was taken as the phase estimate for melatonin.

The curves and phase markers were first analyzed by analysis of variance for repeated measures (rANOVA) with 1, 2, or 3 factors: Condition (advanced vs. fixed sleep), Day (day 2 vs. day 9), and Time of Day. For the CBT curves, the values were binned in 30-min intervals. Statistics for the all curves are presented on *z*-transformed values to account for interindividual amplitude differences. Huynh-Feldt's corrected probabilities (p) are reported for particular Fisher's coefficients and degrees of freedom (F_{df}). Paired *t*-tests were used to locate significant differences. Effect size was calculated to define the size of significant effects (0.3, small; 0.5, medium; 0.8, large) (Cohen, 1988).

RESULTS

Curves

Figure 2 shows the 30-min averaged data for the modified baseline CR protocol on day 2 and for the CR protocol on days 9 and 10 ($n = 10$). During the modified CR protocol at baseline, CBT and subjective energy significantly declined and melatonin and subjective sleepiness increased (factor Time: 1600-2330 h, p at least < 0.01), without any significant variance of mood during this time ($p > 0.30$). The lack of a significant interaction term (Condition × Time) together with a nonsignificant factor condition indicates the similarity of the initial conditions.

In the final CR protocol, all measures showed a significant circadian rhythm (factor Time: 0830-0900 h, p at least < 0.001). Only for the CBT rhythm was a significant interaction term found, supporting the visual impression of a phase advance (Condition × Time: $p < 0.0001$). Therefore, 2-harmonic curve fitting of each individual CBT rhythm was carried out (Brown and Czeisler, 1992) and yielded an estimate of 1.48 ± 0.48 hours difference in phase between the sleep advance and fixed sleep condition ($p = 0.013$). Even though conventional rANOVA analysis of the melatonin rhythm did not yield any significant phase shift, reducing the number of time points to the important decline and rise portion of the curve (from 0830-1130 h and 2030-2330 h) revealed a significant interaction term (Condition × Time: $p = 0.035$), that is, earlier timing of decline and rise after the sleep advance protocol. Both mood and energy self-ratings appeared to decline earlier, and sleepiness appeared to rise earlier in the evening at the end of the advance versus the fixed schedule, but none of these interaction terms were significant when the entire curves were analyzed. Again, by limiting the rANOVA to a shorter time interval (1700-0900 h), a significance (or tendency) was found in the interaction term for sleepiness ($p = 0.057$), energy ($p = 0.026$), and mood ($p = 0.107$). Thus, all 5 measures showed a tendency to, or a significant phase advance, after the advanced versus the fixed schedule.

Sleepiness and Mood Ratings

Subjects felt significantly sleepier (Condition: $p = 0.054$ on absolute values) after the sleep-advancing protocol compared with the fixed sleep schedule (Fig. 2). Therefore, actimetry data of their actual sleep onset time (Fig. 3) were analyzed to check for any possible differences in the amount of sleep (rest). On the last day before the final CR (day 8), sleep onset latency was somewhat longer in the advance schedule than in the fixed schedule (0.65 ± 0.18 h vs. 0.28 ± 0.07 h; $p = 0.09$), according to a linear fit of individual sleep onset times over days 2 through 8. This longer sleep latency suggests that in spite of the similarly scheduled sleep duration of 8.5 h, subjects slept less during the advance protocol. This difference in sleep latency could also be correlated directly with the difference in sleepiness described above ($r = 0.66$, $p = 0.034$, $n = 10$). Thus, a relative sleep deficiency due to a longer time needed to fall asleep during the advance schedule may explain the greater sleepiness at the end of the sleep advance versus control session. In addition, subjects had lower mood ratings after the sleep advance schedule (Fig. 2), which was significant from 0100 to 0230 h ($p = 0.049$) and inversely correlated with the sleepiness score difference ($r = 0.88$, $p = 0.0003$, $n = 10$).

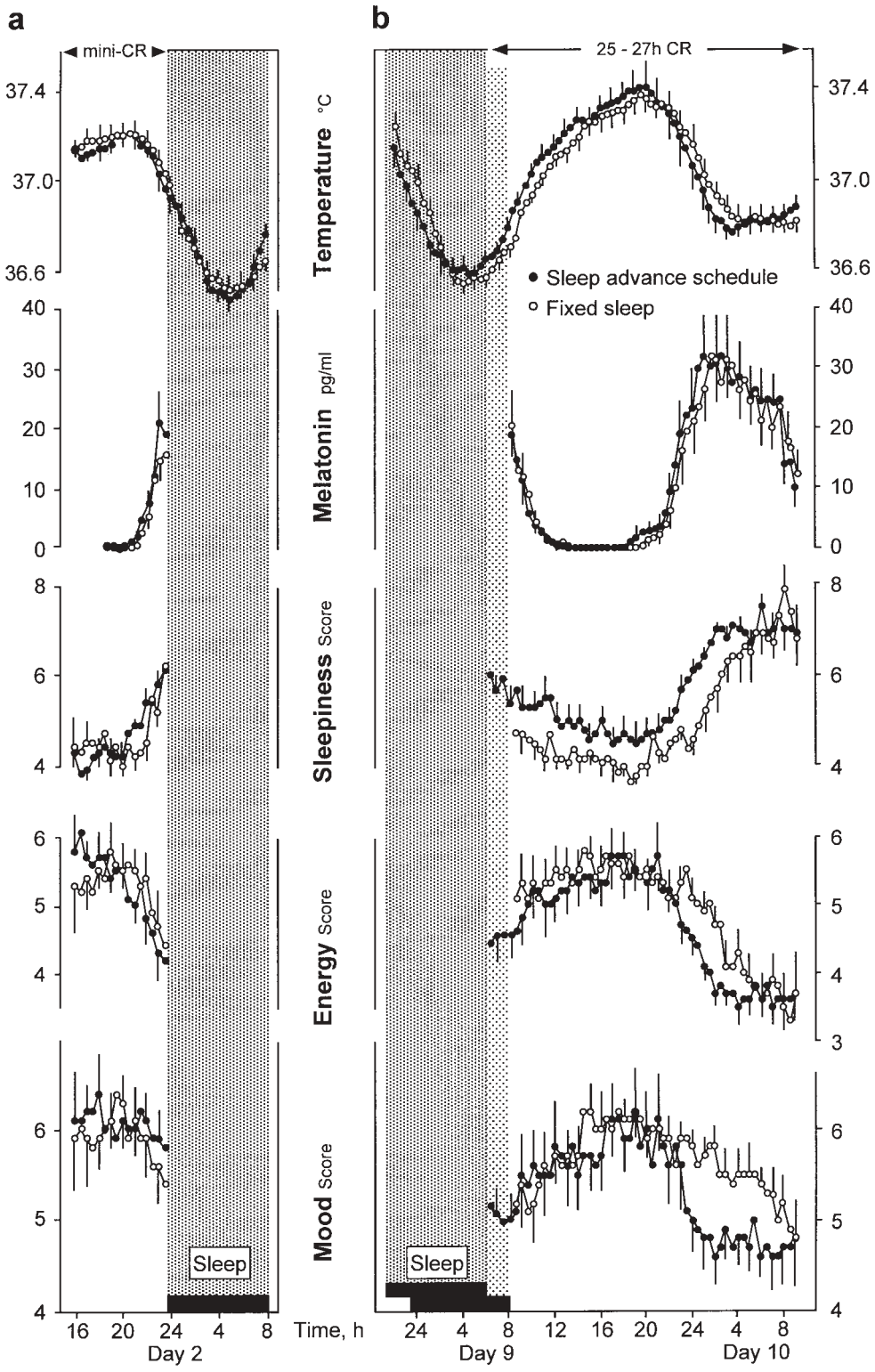

Figure 2. Time course of core body temperature (30-min bins), salivary melatonin, and subjective ratings (sleepiness, energy, and mood) in 10 study participants averaged on day 2 (a) and days 9 and 10 (b) of the advanced and fixed sleep schedule. For visual clarity, only every second SEM has been indicated. Whereas no significant differences were found between the initial curves on day 2, a small phase advance of the curves was seen on day 9 after the advance schedule relative to the fixed sleep schedule (statistics in text). The subjects were sleepier on day 9 of the advance schedule. CR = constant routine.

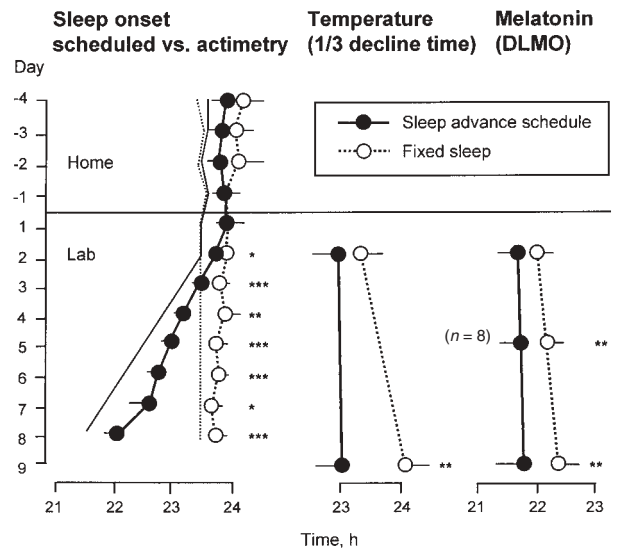

Figure 3. The dynamics of sleep onset and phase marker times during the advanced and fixed sleep schedules ($n = 10$, crossover). The participants followed the scheduled sleep time of 2330 h at home before the experiment quite well (lines without circles [SEM not shown, < 0.13 h]). Sleep onset times were calculated from actimetry data. The difference between the advance and fixed sleep protocols on day 8 reached 2 h by the scheduled sleep time but only 1.63 ± 0.20 h by the achieved sleep onset time. The time of the evening temperature decline (core body temperature 1/3) and melatonin onset (dim light melatonin onset time [DLMO]) drifted (*ns*) to later after the fixed schedule but did not change after the advanced schedule, yielding a relative phase advance (statistics in text). On day 5, $n = 8$ for DLMO, since melatonin did not reach the 3 pg/ml threshold in all subjects by the end of the saliva sampling time.

Phase Markers

Analyses of the phase markers are shown in Figure 3, together with the scheduled and estimated sleep onset. The sleep logs at home documented compliance to the 2330 h lights-off time. The scheduled sleep onset on day 8 attained the 2 h difference, whereas the actual difference in sleep onset by actimetry (by linear fit over days 2-8) was somewhat shorter (1.63 ± 0.20 h, $p = 0.09$).

An rANOVA for the phase markers yielded a significant interaction term Condition × Day for CBT 1/3 decline time ($F_{1,9} = 8.6$, $p = 0.017$) and a tendency for DLMO ($F_{1,9} = 3.6$, $p = 0.091$). That is, the drifts differed between the 2 conditions, being relatively more advanced after the sleep advance when compared with the fixed sleep schedule. The difference between these drifts was significant for CBT (–0.08 h vs. –0.74 h, $p = 0.017$) and tended to significance for melatonin (–0.08 h vs. –0.35 h, $p = 0.091$). The effect size of these relative phase advances were medium for CBT (0.46) and small for melatonin (0.27).

The relative phase advance did not differ significantly between the 2 circadian markers (0.66 h vs. 0.27 h, $p = 0.13$); that is, we cannot say that the CBT rhythm followed the gradual sleep advance more closely than the melatonin rhythm. CBT 1/3 decline time and DLMO were significantly correlated in their change from day 2 to day 9 ($r = 0.74$, $p < 0.0001$, $n = 20$).

The drift of CBT and melatonin phase from days 2 to 9 ranged from +1.62 to –2.56 hours in the fixed sleep and from +3.02 to –2.25 hours in the advanced sleep schedule. The drift in one condition was related to the drift in the other condition ($r = 0.89$, $p = 0.001$ for CBT and $r = 0.88$, $p = 0.002$ for melatonin; $n = 20$), indicating that individuals maintain their pattern of phase drift in near darkness. Irrespective of this individual pattern, the magnitude of the relative phase advance was consistent across subjects.

DISCUSSION

A gradual phase advance of sleep under the most stringently controlled, near-dark (< 0.2 lux), posture, and meal conditions so far tested in humans advanced circadian phase markers of the CBT (significantly) and melatonin rhythms (a tendency), relative to the control condition of fixed sleep. Based on the effect size, however, this effect was small to medium.

Fixed Sleep

The drift of CBT and melatonin phase after 7 days of fixed sleep (2330-0800) under < 0.2 lux ranged from +0.23 to –0.37 h per day. A number of experiments indicate that human circadian rhythms usually do not stably entrain to 24-h days when kept for 6 to 30 days under dim (< 30 lux) light, even with fixed timing for sleep; these studies yielded a range of daily drift of +0.07 to –0.52 h (Nakamura, 1996; Danilenko et al., 2000; Wright et al., 2001), which is similar to that found in our study. The magnitude of phase drift in humans depends on the interaction of intrinsic circadian period and the zeitgeber strength of the photic and nonphotic stimulus (Wright et al., 2001). The drift of human circadian rhythms measured in a forced desynchrony protocol of 20-h or 28-h days has been found to range from +0.37 to –0.78 h per day

(Hiddinga et al., 1997; Carskadon et al., 1999; Czeisler et al., 1999; Wyatt et al., 1999; Wright et al., 2001; Koorengevel et al., 2002).

Sleep Advance

In the sleep advance condition, which corresponded to a 23.67-h day protocol, the range of the individual phase drifts was similar to that in the 24-h day schedule, but the distribution was slightly skewed to the left (i.e., the direction of a phase advance) rather consistently across subjects. The magnitude of this phase advance was significantly less than the imposed 0.33-h daily advance of sleep (by actimetry, the advance of sleep was 0.27 h per day), being 0.10 h a day for the CBT rhythm and 0.04 h a day for DLMO, on average. Shortening the period of the zeitgeber necessarily means that the phase angle between the zeitgeber and endogenous rhythms increase. Longer studies (12-30 days) have also shown that melatonin (and CBT) rhythms failed to entrain completely to a 23.5-h, 23.67-h, or 24.6-h day schedule in dim light (Nakamura, 1996; Miyazaki et al., 2001; Wright et al., 2001). These studies differed from ours in shifting meals and posture with wake-up time as well as changing light between day and night. Since the phase response curve and the zeitgeber strength of these nonphotic stimuli are not yet known, it is not clear how critical their effect may be on the human circadian pacemaker.

Sleep as a Zeitgeber

How could the effects of sleep as zeitgeber be mediated? Is there feedback on the circadian pacemaker in the suprachiasmatic nuclei (SCN)? Brain centers controlling sleep and wakefulness are connected to the SCN (reviewed in Pace-Schott and Hobson, 2002). Circadian information from the SCN reaches hypothalamic structures such as the ventrolateral preoptic nucleus and the dorsal medial hypothalamus, which subserve stage transitions within sleep as well as sleep-wake transitions. The SCN receives inputs from circuits that modulate homeostatic functions (Krout et al., 2002). One feedback may be mediated by arousal. Relaxation induces a large, rapid increase in skin temperatures, and warmer hands and feet are closely correlated with sleepiness (Kräuchi and Wirz-Justice, 2001). Sleep per se has little effect on CBT (Kräuchi and Wirz-Justice, 2001).

In our study, posture was controlled from 21 h in both conditions. Permission to sleep was given earlier in the advance protocol. This meant the subjects relaxed earlier. The earlier vasodilation and heat loss led to an earlier decline in CBT. Although CBT is primarily considered an output rhythm of the light-entrainable circadian pacemaker in the SCN, it is a also a physiologic function regulated in the rest of the body through heat production and heat loss (Kräuchi and Wirz-Justice, 1994). Recent data show that circadian oscillations of ambient temperature may be a zeitgeber for peripheral oscillators without affecting the SCN (Brown et al., 2002). We postulate that changed arousal levels masked CBT and that this very masking could act a zeitgeber for peripheral components of the CBT rhythm.

Such a physiological mechanism may be important for understanding how arousal (common to many nonphotic zeitgebers) can cause phase shifts. It also addresses the discrepancy we observed: An advance of sleep could advance the CBT rhythm but not the SCN-driven rhythm melatonin. When light is the zeitgeber, CBT and melatonin rhythms shift equivalently in parallel (Shanahan and Czeisler, 1991). Other nonphotic zeitgeber studies in humans have also shown some discrepancy. Evening administration of carbohydrate-rich meals phase delayed CBT but not melatonin (Kräuchi et al., 2002). An acoustic signal given in the evening delayed CBT phase, melatonin less so (N. Goel, personal communication, 2002). In the latter study, the converse mechanism may apply: higher alertness means vasoconstriction, less heat loss, and thus a later CBT decline.

Conclusion

The present experiment was rather difficult to carry out, and it is questionable whether the "perfect" experiment is at all possible, that is, keep subjects awake in complete darkness during several days and keep them with minimal activity, closed eyes, and absence of social contacts—but still awake—during the scheduled wake periods in bed. In this ideal protocol, there may indeed be no zeitgeber effect of sleep. Our study does, however, approach such conditions, in that over many days other putative nonphotic zeitgebers were controlled. This permitted us to dissect out the effect of sleep itself on circadian rhythms.

We conclude that sleep per se has little zeitgeber strength to adjust the human circadian clock and that

the main part of the effect previously attributed to sleep may have arisen from concomitant shifts of other nonphotic zeitgebers, in particular, meal times and the thermoregulatory aftereffects of changes in posture, motor activity, and arousal.

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