

Stability, Precision, and Near-24-Hour Period of the Human Circadian Pacemaker

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Regulation of circadian period in humans was thought to differ from that of other species, with the period of the activity rhythm reported to range from 13 to 65 hours (median 25.2 hours) and the period of the body temperature rhythm reported to average 25 hours in adulthood, and to shorten with age. However, those observations were based on studies of humans exposed to light levels sufficient to confound circadian period estimation. Precise estimation of the periods of the endogenous circadian rhythms of melatonin, core body temperature, and cortisol in healthy young and older individuals living in carefully controlled lighting conditions has now revealed that the intrinsic period of the human circadian pacemaker averages 24.18 hours in both age groups, with a tight distribution consistent with other species. These findings have important implications for understanding the pathophysiology of disrupted sleep in older people.

Natural selection has favored endogenous circadian rhythmicity that, in the absence of periodic synchronizing cues from the environment, persists with an intrinsic period close to that of Earth's rotation in nearly all living organisms, including prokaryotes. Clock genes participating in transcriptional-translational feedback loops generate circadian oscillations in plants, insects, and mammals (1, 2), with a period (3–5) that is usually near 24 hours, is highly stable, and exhibits remarkably little interindividual variability within a given species—percent coefficients of variation (PCVs) of only 0.08% in the kangaroo rat, 0.3% in hamsters, 0.54% in the gila monster, and 0.7% in mice (3, 4, 6). An age-related shortening of circadian period, which is a determinant of the phase angle of entrainment, has been hypothesized to account for the circadian phase advance and early-morning awakening observed frequently in the elderly (7–11).

Quantification of circadian period in humans has yielded inconsistent results. Although the free-running circadian period of the human activity rhythm was believed to average more than 25 hours, as was initially reported nearly 40 years ago (12), it has since been reported to vary from 13 to 65 hours in normal subjects,

with a PCV of 30.3% (13). The average free-running circadian period of the human body temperature rhythm has been reported to vary with both the experimental environment and the subjects' behavior, ranging from 24.2 to 25.1 hours (13–15). However, the generality of these findings has been limited by reports that activity (16, 17), knowledge of time of day (18), and exposure to ordinary indoor room light (19, 20) can shift circadian phase or alter the observed free-running circadian period in humans and thus may have influenced those observations (21). Here, we assessed the intrinsic period of the circadian pacemaker in 24 young and older human subjects, each living for approximately 1 month in an environment free of time cues under conditions of controlled exposure to the light-dark cycle on a forced desynchrony protocol pioneered by Kleitman more than 60 years ago (22), using methodology detailed elsewhere (21, 23).

We studied 11 healthy young men (mean age 23.7 years) and 13 healthy older subjects (9 men and 4 women; mean age 67.4 years) for 29 to 38 days (24). During the forced desynchrony protocol, the bedtime of each subject was scheduled to occur 4 hours later each day for ~3½ weeks. Each subject's sleep-wake cycle was thus scheduled to a 28-hour "day" (Fig. 1). Rhythms driven by the circadian pacemaker were thereby desynchronized from each subject's sleep-wake cycle. In this way, exposure to both photic and nonphotic (25, 26) synchronizers linked to the scheduled sleep-wake cycle was distributed evenly across all circadian phases (21). The 28-hour day length on this forced desynchrony protocol was (i) far enough outside the range of entrainment of the human

- struction of a putative full-length coding region (608 codons) for CARM1 (GenBank accession number AF117887).
11. J. D. Gary and S. Clarke, *Prog. Nucleic Acids Res. Mol. Biol.* **61**, 65 (1998).
 12. J. Najbauer, B. A. Johnson, A. L. Young, D. W. Aswad, *J. Biol. Chem.* **268**, 10501 (1993).
 13. W.-J. Lin, J. D. Gary, M. C. Yang, S. Clarke, H. R. Herschman, *ibid.* **271**, 15034 (1996).
 14. D. W. Aswad, B. T. Schurter, S. S. Koh, unpublished data.
 15. D. Chen, H. Ma, S. S. Koh, M. R. Stallcup, unpublished data.
 16. A. Imhof et al., *Curr. Biol.* **7**, 689 (1997).
 17. K. Luger and T. J. Richmond, *Curr. Opin. Genet. Dev.* **8**, 140 (1998).
 18. J. M. Aletta, T. R. Cimato, M. J. Ettinger, *Trends Biochem. Sci.* **23**, 89 (1998).
 19. H. Hong, K. Kohli, A. Trivedi, D. L. Johnson, M. R. Stallcup, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 4948 (1996).
 20. S. T. Crews, *Genes Dev.* **12**, 607 (1998).
 21. J. Tang, J. D. Gary, S. Clarke, H. R. Herschman, *J. Biol. Chem.* **273**, 16935 (1998); H. S. Scott et al., *Genomics* **48**, 330 (1998); J. D. Gary, W.-J. Lin, M. C. Yang, H. R. Herschman, S. Clarke, *J. Biol. Chem.* **271**, 11585 (1996).
 22. H. Hong, K. Kohli, M. J. Garabedian, M. R. Stallcup, *Mol. Cell. Biol.* **17**, 2735 (1997).
 23. Plasmid construction. Mammalian cell expression vectors: pSG5.HA was constructed by inserting a synthetic sequence coding for a translation start signal, hemagglutinin A (HA) tag, Eco RI site, and Xho I site into the Eco RI-Bam HI site of pSG5 (Stratagene), which has SV40 and T7 promoters. The original Eco RI site was destroyed by this insertion, but the Bam HI site was preserved, leaving a multiple cloning site after the HA tag containing Eco RI, Xho I, Bam HI, and Bgl II sites. The following protein-coding regions were cloned into pSG5.HA, in frame with the HA tag, with the indicated insertion sites: GRIP1_{5–1462} (full length) and CARM1_{3–608} (full length) at the Eco RI site; GRIP1_{5–765} at the Eco RI-Xho I site; GRIP1_{730–1121} and GRIP1_{1121–1462} were Eco RI-Sal I fragments inserted at the Eco RI-Xho I site; SRC-1a_{1–1441} (full length) was a Sma I-Sal I fragment inserted at the Eco RI site, which was blunted by filling with Klenow polymerase, and the Xho I site. Expression vectors for Gal4DBD fused to various GRIP1 fragments were constructed by inserting the appropriate fragments into pM (Clontech) as follows: GRIP1_{122–1462}, Eco RI-Bgl II fragment inserted into Eco RI-Bam HI site; GRIP1_{563–1121} and GRIP1_{5–765}, Eco RI-Sal I fragments inserted into homologous site. Vectors for GST fusion proteins were constructed in pGEX-4T1 (Pharmacia): for GST-CARM1 the original 3.2-kb Eco RI fragment from pGAD10.CARM1 was inserted; for GST-GRIP1_C (amino acids 1122 to 1462) an Eco RI-Sal I fragment was inserted. The CARM1 VLD-to-AAA mutation was engineered with the Promega Gene Editor Kit.
 24. J. P. Chamberlin, *Anal. Biochem.* **98**, 132 (1978).
 25. A. O. Brinkmann et al., *J. Steroid Biochem. Mol. Biol.* **34**, 307 (1989).
 26. S. Green, I. Isseman, E. Sheer, *Nucleic Acids Res.* **16**, 369 (1988).
 27. W. Feng et al., *Science* **280**, 1747 (1998).
 28. K. Umehono and R. M. Evans, *Cell* **57**, 1139 (1989).
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circadian pacemaker so as to minimize the influence of the imposed schedule on the observed circadian period (21, 27, 28), and (ii) imposed consistently throughout the protocol. This was done to avoid the artifactual extension of the range of entrainment associated with the fractional desynchronization protocol (29), in which a gradually lengthening light-dark schedule was imposed (13). Also, to minimize the circadian resetting effects of ambient light (19, 28), we maintained constant low light levels during the scheduled wake episodes (Fig. 1). Several subjects returned for additional month-long studies so that we could compare the results of the forced desynchrony protocol with those of the classical free-running protocol (30).

Core body temperature, plasma melatonin, and plasma cortisol were sampled during the forced desynchrony protocols. Endogenous circadian period was estimated using a non-orthogonal spectral analysis (NOSA) technique, in which these data were fitted simultaneously with periodic components corresponding to both the forced period of the imposed sleep-wake cycle and the sought-for period of the endogenous circadian rhythm, together with their harmonics, using an exact maximum likelihood fitting procedure (31, 32).

The estimated intrinsic periods of the core body temperature, melatonin, and cortisol rhythms were highly correlated when analyzed within an individual subject (Table 1) (33), which supports the hypothesis that the circadian

period measured in these studies reflects the intrinsic period of a central circadian pacemaker. Therefore, our estimate of the intrinsic period of the circadian pacemaker for each subject was computed by averaging the period estimates derived from each available variable. These intrinsic circadian period estimates from the 24 subjects were narrowly distributed, with nearly 90% of the estimates between 24.00 and 24.35 hours (Fig. 2). The average estimated (\pm SEM) intrinsic period was 24.18 ± 0.04 hours (PCV 0.54%) in the young men and 24.18 ± 0.04 hours (PCV 0.58%) in the older subjects (see Table 1).

The intrinsic period we observed does not appear to have been dependent on the length of the imposed sleep-wake cycle. The intrinsic period of the core body temperature rhythm derived from subjects 1111 and 1507 on both a 20- and a 28-hour forced desynchrony study were nearly equivalent: 24.29 and 24.28 hours, respectively, for subject 1111 (see Fig. 1) and 24.26 and 24.16 hours, respectively, for subject 1507. Estimates of the intrinsic period of the core body temperature data alone from the combined group of 24 subjects on the 28-hour forced desynchrony protocol (mean \pm SEM = 24.17 ± 0.03 hours) and from a series of 14 subjects studied on a 20-hour forced desynchrony protocol from two other experiments (mean \pm SEM = 24.15 ± 0.04 hours) (34) were not significantly different ($P = 0.6211$). One older subject (1507) also participated in a

42.85-hour forced desynchrony protocol and exhibited a temperature period of 24.15 hours, as compared to a period of 24.16 hours on the 28-hour forced desynchrony protocol. These results are also consistent with the 24.20-hour temperature period estimate of an additional young man (1134) who participated in an 11-hour forced desynchrony experiment. Thus, the observed circadian period was equivalent on various imposed sleep-wake and associated light-dark cycles (11, 20, 28, or 42.85 hours). In contrast, when two of the same subjects participated in classical free-running studies in which they self-selected their exposure to a light-dark cycle (light, ~ 150 lux; dark, <0.03 lux), the observed period of the temperature cycle was substantially longer [subject 1111, 25.1 hours (Fig. 1); subject 1105, 25.0 hours].

We hypothesize that the longer, more variable circadian period of the temperature rhythm observed in such classical free-running protocols (35) [averaging 25.1 hours (PCV 2.5%) among free-running subjects whose activity-rest cycle was synchronized with their body temperature rhythm and 24.9 hours (PCV 0.8%) among internally desynchronized subjects (13)] occurs because both synchronized and spontaneously desynchronized free-running subjects preferentially select room light exposure before the circadian temperature minimum, and darkness after that minimum (36, 37), thereby systematically eliciting light-induced phase delays and minimizing light-in-

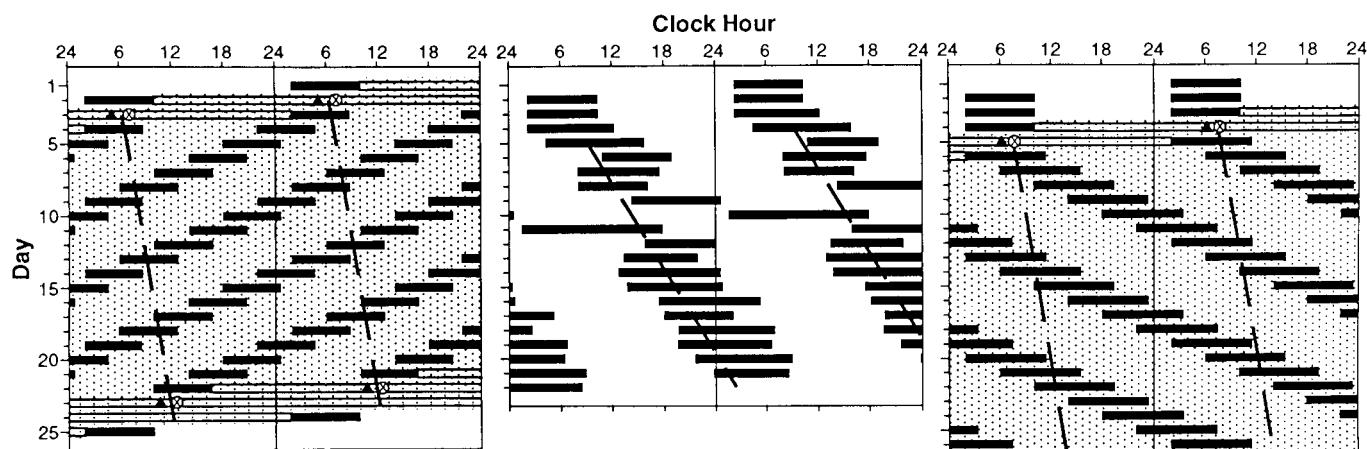


Fig. 1. Experimental results from a 22-year-old man (subject 1111) living in an environment free of time cues on a 20-hour forced desynchrony protocol (left panel), a classical free-running protocol (center panel), and a 28-hour forced desynchrony protocol (right panel). The rest-activity cycle is plotted in a double raster format, with successive days plotted both next to and beneath each other and clock hour indicated on the abscissa. Baseline sleep episodes were scheduled at their habitual times (based on an average of their schedule during the week before laboratory admission). Thereafter, sleep/dark episodes (solid bars, light intensity <0.03 lux) were scheduled for 6.67 hours (33% of imposed day) in the 20-hour protocol, self-selected by subject (averaging 28% of cycle) in the free-running protocol, and scheduled for 9.33 hours (33% of imposed day) in the 28-hour protocol. During wake episodes, the light intensity was ~ 15 lux (20- and 28-hour protocols) or ~ 150 lux (free-running protocol). Constant routines (open bars) for phase assessments of the endogenous circadian temperature nadir (\otimes) and the fitted melatonin maximum (\blacktriangle) were conducted before and after forced desynchrony in all subjects except 1209, who began forced desynchrony immediately after the three baseline days. Period estimations were performed with the use of temperature data (continuously collected via rectal thermistor throughout all studies) and plasma melatonin and cortisol data (assayed from samples collected every 20 to 60 min during segments of the study in the 20- and 28-hour protocols). The estimated phase of the circadian temperature rhythm (dashed line) was determined by nonorthogonal spectral analysis (31, 32). The temperature period estimates are nearly equivalent under both forced desynchrony protocols (20-hour protocol, 24.29 hours; 28-hour protocol, 24.28 hours), independent of the imposed rest-activity cycle. However, the estimated temperature period (25.07 hours) observed during free-running conditions (with self-selected rest-activity cycle averaging 27.07 hours) was much longer.

REPORTS

duced phase advances (28, 38). We thus hypothesize that this unequal distribution of the sleep-wake and associated light-dark cycle across circadian phases in the free-running protocol (as compared with their more equal distribution in the forced desynchrony protocol) was responsible for the overestimation of circadian period derived from the free-running protocol. This hypothesis is supported by (i) the results of simulations using Kronauer's mathematical model of the resetting effect of light on the human circadian pacemaker, which indicate that such feedback effects of ordinary room light alone can lengthen the apparent circadian period observed under classical free-running conditions by more than 0.7 hours (28); (ii) the observation of a shorter average endogenous circadian temperature period derived from free-running subjects when their rest-activity cycle spontaneously desynchronizes from their body temperature cycle, and thereby distributes light exposure more evenly across all circadian phases (13, 28, 36, 39); and (iii) the results of subjects 1105 and 1111, who each exhibited a much longer apparent circadian period when studied on the classical free-running protocol than when studied on the forced desynchrony protocol in dim light.

Unlike the highly variable, much longer circadian period estimates derived from body temperature data in classical free-running human studies, the much smaller coefficient of variation (PCV 0.55%) and the nearer-to-24-hour mean value (24.18 hours) of the intrinsic circadian period estimates derived from all three variables in these forced desynchrony studies is consistent with coefficients of variation and mean values for circadian period estimates observed in other mammals and derived from mathematical modeling of data from human circadian studies (28, 40). These results suggest that the intrinsic period of the human circadian

pacemaker is likely to be under the same tight genetic control as has been demonstrated for a wide variety of other species (1, 3–5). Precise estimation of the circadian period is critical for pursuing the possible genetic basis of circadian rhythm sleep disorders.

Our results on the forced desynchrony protocol, together with those of others (14, 15, 41), are in contrast to those of Wever, who observed an average circadian temperature period of 24.8 hours in subjects living in constant conditions in whom internal desynchronization was forced by an imposed 20-, 28-, 30-, or 32-hour cycle of ordinary room light alternating with absolute darkness (13). However, naps around the time of the temperature nadir (42), which are associated with reduced retinal light exposure, may have exerted feedback effects that influenced the estimate of the average circadian period (28), because in that study the timing of sleep was not restricted to the scheduled dark episodes.

Although the group average period estimate from our series is similar to that recently reported using a forced desynchrony protocol of only 5 days in duration (41), the interindividual variability of circadian period estimates derived from that much shorter protocol was significantly greater than it was for estimates derived from our 3- to 4-week protocol (F test, $P < 0.0001$), with more than half of the period estimates from that study outside the 95% confidence interval of the present study (41, 43).

Even though none of the subjects in our experiments were allowed to nap, we observed a consistent period averaging 24.18 hours, contrary to a prior report of a 24.7-hour circadian period in non-napping subjects (14). We hypothesize that this discrepancy was observed because the sleep episodes of the non-napping subjects in that earlier report were not evenly distributed across circadian phases (14) and were therefore apt to induce feedback effects on the pacemaker (25, 28).

Table 1. Intrinsic periods of the temperature (τ_t), melatonin (τ_m), and cortisol (τ_c) rhythms (expressed as hours:minutes) in young and older subjects in the 28-hour forced desynchrony protocol. For each subject, the estimated period of each of the three rhythms lies within the 95% confidence interval of the other two rhythms. τ_t , τ_m , and τ_c were highly correlated [Pearson correlation: τ_t versus τ_m , $r = 0.951$; τ_t versus τ_c , $r = 0.982$; τ_m versus τ_c , $r = 0.984$ ($P < 0.0001$ in all cases)]. Our composite estimate of the intrinsic period for each subject (τ) was computed by averaging τ_t , τ_m , and τ_c , if available. Constraints on the total blood collection volume and vascular access limited the number of older subjects for whom cortisol and melatonin data were available; also, in two young subjects (1145 and 1257), an inadequate number of blood samples were collected and analyzed for cortisol concentrations to obtain a reliable estimate of circadian period.

Subject	Age (years)	Sex	τ_t (\pm SD)	τ_m (\pm SD)	τ_c (\pm SD)	τ
<i>Young subjects</i>						
1105	25	M	24:16 \pm :02	24:14 \pm :05	24:17 \pm :10	24:16
1106	21	M	24:14 \pm :01	24:14 \pm :02	24:18 \pm :07	24:16
1111	22	M	24:17 \pm :01	24:17 \pm :03	24:19 \pm :05	24:18
1120	25	M	24:08 \pm :01	24:09 \pm :01	24:10 \pm :07	24:09
1122	23	M	24:09 \pm :01	24:07 \pm :02	24:08 \pm :09	24:08
1133	23	M	23:53 \pm :01	23:51 \pm :03	23:52 \pm :10	23:52
1136	22	M	24:09 \pm :01	24:10 \pm :04	24:13 \pm :07	24:11
1144	23	M	24:15 \pm :01	24:17 \pm :01	24:18 \pm :06	24:16
1145	30	M	24:09 \pm :01	24:11 \pm :04	—	24:10
1209	21	M	24:06 \pm :01	24:05 \pm :00	24:08 \pm :01	24:07
1257	26	M	24:19 \pm :02	24:23 \pm :01	—	24:21
Range	21–30		23:53–24:19	23:51–24:23	23:52–24:19	23:52–24:21
Mean	23.7		24:10	24:11	24:11	24:11
\pm SD	2.7		00:07	00:08	00:09	00:08
\pm SEM	0.8		00:02	00:03	00:03	00:02
<i>Older subjects</i>						
1213	74	F	24:02 \pm :02	—	—	24:02
1215	64	M	24:07 \pm :07	24:01 \pm :02	24:07 \pm :08	24:05
1304	64	M	24:03 \pm :03	24:09 \pm :07	—	24:06
1319	67	F	24:10 \pm :02	24:10 \pm :04	—	24:10
1355	69	M	24:25 \pm :02	24:25 \pm :03	—	24:25
1366	66	M	24:28 \pm :05	24:30 \pm :03	—	24:29
1375	66	M	24:19 \pm :02	24:20 \pm :05	—	24:20
1458	67	M	24:09 \pm :02	24:13 \pm :03	—	24:11
1475	72	F	24:00 \pm :03	24:04 \pm :07	—	24:02
1485	65	M	24:06 \pm :01	24:09 \pm :05	—	24:07
1490	71	F	24:13 \pm :02	—	—	24:13
14A6	65	M	24:04 \pm :02	24:07 \pm :02	—	24:05
1507	66	M	24:10 \pm :03	—	—	24:10
Range	64–74		24:00–24:28	24:01–24:30	—	24:02–24:29
Mean	67.4		24:10	24:13	—	24:11
\pm SD	3.2		00:09	00:09	—	00:08
\pm SEM	0.2		00:02	00:03	—	00:02

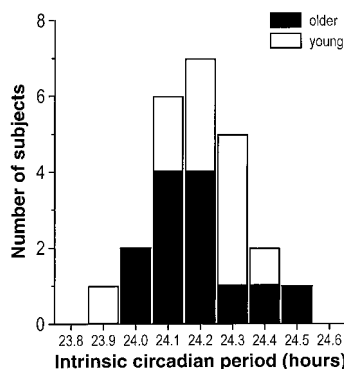


Fig. 2. Histogram of intrinsic circadian period (τ) estimates derived from young and older subjects. Intrinsic circadian period estimates of older subjects are indicated by solid bars, those of young subjects by open bars. Each subject's estimated intrinsic circadian period is reported as the average of the estimated periods from his or her core body temperature, melatonin, and cortisol rhythms (see Table 1).

Interestingly, scheduling subjects to a non-24-hour rest-activity cycle alone is not in itself sufficient to assess the intrinsic circadian period in human subjects: The body temperature cycle of subjects scheduled to a 27-hour rest-activity cycle, but not shielded from exposure to Earth's 24-hour light-dark cycle, remained entrained to the 24.0-hour day (44). However, lack of knowledge of the time of day may not be so critical when a non-24-hour schedule is behaviorally imposed. Estimates of the endogenous circadian period of the melatonin rhythm (24.27 hours, PCV 0.84%) derived from a field study of submariners living undersea (and hence shielded from bright outdoor but not artificial light) for 6 weeks while maintaining an 18-hour naval duty schedule were only about 0.1 hour longer than the results reported here from subjects studied in our controlled laboratory environment (45), even though the submariners knew the time of day and only the work hours (but not the sleep or meal times) were scheduled to an 18-hour routine in that field study.

The circadian period of blind subjects not entrained to the 24-hour day while they are living in society has been reported to average 24.3 to 24.5 hours (26, 46), somewhat longer than we now report for sighted subjects. This apparent discrepancy may be due to (i) the influence of the nonuniform distribution of non-photic synchronizers associated with the self-selected rest-activity cycle of blind subjects [and of sighted subjects living in constant darkness (13)], which has been shown to affect circadian period estimates in other mammals living in constant darkness (25); (ii) the inclusion in the group average of only those blind subjects with longer than average circadian periods who were unable to maintain entrainment via weaker non-photic synchronizers (25, 26), coupled with the classification of all blind subjects whose period estimates were indistinguishable from 24 hours as entrained, resulting in their exclusion from the group average; or (iii) aftereffects of entrainment to the 24-hour day in the sighted subjects (47). The final possibility would suggest that prior entrainment to the 24-hour day in sighted people might shorten the circadian period observed upon release from entrainment.

In the present experiment, contrary to a prior assessment of the temperature rhythm under classical free-running conditions (8), we did not detect a significant difference in the intrinsic circadian period between the healthy young and older subjects studied; the average intrinsic period (\pm SEM) in the young men was 24.18 ± 0.04 hours, versus 24.18 ± 0.04 hours in the older men and women ($P = 0.961$) (Table 1), consistent with recent reports in both male and female Syrian hamsters studied throughout their life-span (48). However, with the number of subjects we studied and the observed variability in the intrinsic period, we only had the power

(presumed $\alpha = 0.05$; power = 0.90; standard deviation = 0.15 hours) to detect a difference in circadian period greater than 9 min between the young and older subjects in our study. Given our estimates of the distribution of circadian periods in young and older subjects seen in Fig. 2, it remains possible that a much larger series of such studies might detect a small age-related difference in average circadian period.

Despite comparable circadian periods, the older subjects in this study exhibited the characteristically earlier entrained circadian phase and earlier morning awakening typically found in this age group relative to young subjects (23). Therefore, it is unlikely that the systematic age-related advance in circadian phase and the time of spontaneous awakening can be attributed—at least in this healthy group—to an age-related shortening of circadian period (7–11). The recent report of a similar estimate of circadian period in adolescents (49) further supports the conclusion that this pacemaker property remains stable with age. Putative mechanisms for age-related changes in sleep-wake timing and consolidation include age-related changes in the sleep-homeostatic process and its interaction with the circadian and entrainment processes (23). However, these results do not preclude the possibility that abnormal circadian entrainment might be due to an abnormal circadian period in some older individuals, as has been reported (10).

These results contribute to understanding circadian entrainment in both young and older people and have practical implications for understanding the pathophysiology of, and developing treatments for, circadian rhythm sleep disorders, including the dysomnia of night shift work, transmeridian travel, both delayed and advanced sleep phase syndrome, and disrupted sleep in older people. These data reveal that the human circadian pacemaker is as stable and precise in measuring time as that of other mammals, and they suggest that understanding of the molecular mechanisms regulating circadian period in other species may well apply to humans (50).

References and Notes

1. D. P. King et al., *Cell* **89**, 641 (1997).
2. Z. S. Sun et al., *ibid.* **90**, 1003 (1997); H. Tei et al., *Nature* **389**, 512 (1997); L. P. Shearman, M. J. Zylka, D. R. Weaver, L. F. Kolakowski, S. M. Reppert, *Neuron* **19**, 1261 (1997); N. Gekakis et al., *Science* **280**, 1564 (1998); M. J. Zylka, L. P. Shearman, D. R. Weaver, S. M. Reppert, *Neuron* **20**, 1103 (1998); S. K. Crosthwaite, J. C. Dunlap, J. J. Loros, *Science* **276**, 763 (1997); T. K. Darlington et al., *ibid.* **280**, 1599 (1998); H. Hao, D. L. Allen, P. E. Hardin, *Mol. Cell. Biol.* **17**, 3687 (1997); C. Liu, D. R. Weaver, S. H. Strogatz, S. M. Reppert, *Cell* **91**, 855 (1997); J. E. Rutilla et al., *ibid.* **93**, 805 (1998); S. M. Reppert, *Neuron* **21**, 1 (1998); J. C. Dunlap, *Science* **280**, 1548 (1998).
3. M. R. Ralph and M. Menaker, *Science* **241**, 1225 (1988).
4. M. H. Vitaterna et al., *ibid.* **264**, 719 (1994).
5. R. J. Konopka and S. Benzer, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2112 (1971); J. J. Loros, S. A. Denome, J. C. Dunlap, *Science* **243**, 385 (1989); J. C. Dunlap, *Annu. Rev. Physiol.* **55**, 683 (1993); T. Kondo et al., *Science*

- 266**, 1233 (1994); A. J. Millar, I. A. Carré, C. A. Strayer, N.-H. Chua, S. A. Kay, *ibid.* **267**, 1161 (1995).
6. C. H. Lowe, D. S. Hinds, P. J. Lardner, K. E. Justice, *Science* **156**, 531 (1967).
7. C. S. Pittendrigh and S. Daan, *ibid.* **186**, 548 (1974); L. P. Morin, *J. Biol. Rhythms* **3**, 237 (1988).
8. E. D. Weitzman, M. L. Moline, C. A. Czeisler, J. C. Zimmerman, *Neurobiol. Aging* **3**, 299 (1982).
9. W. C. Dement et al., in *The Biology of Aging*, C. E. Finch and E. L. Schneider, Eds. (Van Nostrand Reinhold, New York, 1985), pp. 692–717; B. L. Myers and P. Badia, *Neurosci. Biobehav. Rev.* **19**, 553 (1995).
10. C. A. Czeisler et al., *Science* **233**, 667 (1986).
11. C. A. Czeisler et al., *Lancet* **340**, 933 (1992).
12. J. Aschoff and R. Wever, *Naturwissenschaften* **49**, 337 (1962).
13. R. A. Wever, *The Circadian System of Man: Results of Experiments Under Temporal Isolation* (Springer-Verlag, New York, 1979); *Experientia* **40**, 1226 (1984).
14. S. S. Campbell, D. Dawson, J. Zulley, *Sleep* **16**, 638 (1993).
15. B. Middleton, J. Arendt, B. M. Stone, *J. Sleep Res.* **5**, 69 (1996).
16. O. M. Buxton et al., *Am. J. Physiol.* **273**, E536 (1997); R. Leproult, O. Van Reeth, M. M. Byrne, J. Sturis, E. Van Cauter, *J. Biol. Rhythms* **12**, 245 (1997).
17. O. Van Reeth et al., *Am. J. Physiol.* **266**, E964 (1994); C. I. Eastman, E. K. Hoese, S. D. Youngstedt, L. Liu, *Physiol. Behav.* **58**, 1287 (1995).
18. J. N. Mills, *J. Physiol. (London)* **174**, 217 (1964).
19. D. B. Boivin, J. F. Duffy, R. E. Kronauer, C. A. Czeisler, *Nature* **379**, 540 (1996); D. B. Boivin and C. A. Czeisler, *Neuroreport* **9**, 779 (1998).
20. J. Waterhouse et al., *Neurosci. Lett.* **245**, 97 (1998).
21. C. A. Czeisler, J. S. Allan, R. E. Kronauer, *Sleep Res.* **15**, 266 (1986); in *Sleep and Biological Rhythms: Basic Mechanisms and Applications to Psychiatry*, J. Montplaisir and R. Godbout, Eds. (Oxford Univ. Press, New York, 1990), pp. 87–98.
22. N. Kleitman, *Sleep and Wakefulness* (Univ. of Chicago Press, Chicago, 1939).
23. J. F. Duffy, D.-J. Dijk, E. B. Klerman, C. A. Czeisler, *Am. J. Physiol.* **275**, R1478 (1998); D.-J. Dijk, J. F. Duffy, E. Riel, T. L. Shanahan, C. A. Czeisler, *J. Physiol. (London)* **516**, 611 (1999).
24. Subjects had no medical, psychiatric, or sleep disorders, as determined by history, physical examination, electrocardiogram, clinical laboratory screening tests, psychological questionnaires, interview with a clinical psychologist, and, for older subjects, chest radiograph and diagnostic sleep recording. None reported regular night or rotating shift work within the prior 3 years, or crossing more than one time zone within the prior 3 months. Urinary toxicological screening was used to verify that all subjects were drug-free at the time of study. The experimental procedures were reviewed and approved by the Human Research Committee at Brigham and Women's Hospital, and each subject gave written informed consent (23).
25. D. M. Edgar, C. E. Martin, W. C. Dement, *J. Biol. Rhythms* **6**, 185 (1991); O. Van Reeth and F. W. Turek, *Nature* **339**, 49 (1989); D. Janik and N. Mrosovsky, *Physiol. Behav.* **53**, 431 (1993); N. Mrosovsky, S. G. Reeb, G. I. Honrado, P. A. Salmon, *Experientia* **45**, 696 (1989); E. Van Cauter et al., *J. Biol. Rhythms* **8**, S99 (1993); S. Amir and J. Stewart, *Nature* **379**, 542 (1996).
26. E. B. Klerman et al., *Am. J. Physiol.* **43**, R991 (1998).
27. R. E. Kronauer, J. E. Fookson, S. H. Strogatz, *Sleep Res.* **15**, 274 (1986).
28. R. E. Kronauer and C. A. Czeisler, in *Light and Biological Rhythms in Man*, L. Wetterberg, Ed. (Pergamon, Oxford, 1993), pp. 217–236; C. A. Czeisler et al., *Science* **244**, 1328 (1989); E. B. Klerman, D.-J. Dijk, R. E. Kronauer, C. A. Czeisler, *Am. J. Physiol.* **270**, R271 (1996).
29. P. H. Gander, R. E. Kronauer, C. A. Czeisler, M. C. Moore-Ede, *Am. J. Physiol.* **247**, R427 (1984).
30. Two young subjects (1111 and 1105) returned for a classical free-running study in which they self-selected their light-dark cycle (12, 13) (Fig. 1, center panel). Two subjects (1111 and 1507) later returned for a 20-hour forced desynchrony protocol (Fig. 1, left panel). One of those subjects (1507) also returned for yet another forced desynchrony protocol with a

42.85-hour imposed day length. The ratio of scheduled bedtime to scheduled wake time was maintained at 1:2 for each forced desynchrony protocol.

31. Using the NOSA technique, core-temperature data collected throughout the month-long experiment were modeled as a harmonic regression model with continuous first-order autoregressive [AR(1)] noise defined as

$$y_t = s_t + x_t + v_t \quad (1)$$

where y_t is the core-temperature measurement at time t , s_t is the circadian signal, x_t is the forced desynchrony component, and v_t is the AR(1) noise. We define

$$s_t = \mu + \sum_{r=1}^2 A_r \cos\left(\frac{2\pi r t}{\tau}\right) + B_r \sin\left(\frac{2\pi r t}{\tau}\right) \quad (2a)$$

$$x_t = \sum_{k=1}^8 C_k \cos\left(\frac{2\pi k t}{28}\right) + D_k \sin\left(\frac{2\pi k t}{28}\right) \quad (2b)$$

$$v_t = \exp(-\alpha\Delta)v_{t-1} + \varepsilon_t \quad (2c)$$

where μ is mean temperature, τ is the intrinsic period of the circadian pacemaker, A_r and B_r are respectively the cosine and sine coefficients of the r^{th} harmonic of the circadian signal, C_k and D_k are respectively the cosine and sine coefficients of the k^{th} harmonic of the forced desynchrony component, Δ is the sampling interval, α is the approximate time constant of the thermoregulatory system, and the ε_t 's are independent, identically distributed Gaussian noise with zero mean and variance σ_ε^2 . The variance of v_t is $\sigma_v^2 = \sigma_\varepsilon^2 [1 - \exp(-2\alpha\Delta)]^{-1}$. The choice of two harmonics to model the circadian component is based on Brown and Czeisler (32), whereas the choice of eight harmonics to model the forced desynchrony component was determined empirically. The model was fit to the data by an exact maximum likelihood method [R. H. Jones, *Longitudinal Data with Serial Correlation: A State-Space Approach* (Chapman & Hall, New York, 1993); E. N. Brown and C. H. Schmid, in *Methods in Enzymology, Numerical Computer Methods*, Part B, L. Brand and M. L. Johnson, Eds. (Academic Press, Orlando, FL, 1994), pp. 171–181]. The standard deviation of the period estimate used to compute the 95% confidence intervals for τ was computed as

$$\sigma_\tau = \left\{ \frac{6\tau^4 \sigma_\varepsilon^2 \Delta [1 - \exp(-2\alpha\Delta)]}{\pi^2 T^3 \sum_{r=1}^2 \mu^2 (A_r^2 + B_r^2) [1 - 2\exp(-\alpha\Delta) \cos\left(\frac{2\pi r \Delta}{\tau}\right) + \exp(-2\alpha\Delta)]} \right\}^{1/2} \quad (3)$$

where T is the study length [E. N. Brown, V. Solo, Y. Choe, Z. Zhang, *Tech. Rep. 95-01* (Statistics Research Laboratory, Department of Anesthesia and Critical Care, Massachusetts General Hospital, April 1996; revised November 1997)]. The hormone measurements have no thermoregulatory component; hence, for their analyses, we used the model in Eq. 2 with $v_t = \varepsilon_t$, and the formula in Eq. 3 with $\alpha = \infty$. For the free-running studies, only core body temperature was sampled and the fitting did not include a forced period. NOSA can include periodic terms that may arise from nonlinear interactions between the basic periodic signals s_t and x_t . We have explored such additional terms and identified those that rise above the level of noise in the temperature data. However, when these are included, the effect on the average endogenous period reported here is minimal (<1 min) and not statistically significant. We therefore report the endogenous periods estimated without interaction periodicities, which can be used to investigate related nonlinear processes that are beyond the scope of this report.

32. E. N. Brown and C. A. Czeisler, *J. Biol. Rhythms* **7**, 177 (1992).
33. T. L. Shanahan and C. A. Czeisler, *Sleep Res.* **20A**, 557 (1991); T. L. Shanahan, thesis, Harvard Medical School (1995).
34. J. K. Wyatt et al., *Sleep Res.* **26**, 759 (1997); D. W. Rimmer, D. J. Dijk, J. K. Wyatt, D. F. Dinges, C. A. Czeisler, *Med. Sci. Sports Exercise* **30**, 5 (1997).

35. Free-running subjects, who self-selected the timing of their sleep-wake and light-dark schedule while living in an environment free of time cues, chose sleep episode fractions averaging 29 to 33% (8, 12, 13, 36, 37, 39), comparable to the sleep fraction imposed in the forced desynchrony reported herein.
36. C. A. Czeisler, E. D. Weitzman, M. C. Moore-Ede, J. C. Zimmerman, R. S. Knauer, *Science* **210**, 1264 (1980).
37. J. Zulley, R. Wever, J. Aschoff, *Pfluegers Arch.* **391**, 314 (1981).
38. This same mechanism by which free-running subjects exhibit a longer observed period than their actual intrinsic circadian period may underlie some clinical cases of non-24-hour sleep-wake schedule disorder in which subjects exhibit an average sleep-wake cycle duration of ≥ 25 hours, even though their actual intrinsic circadian period may be much closer to 24 hours [J. S. Emens, D. J. Brotman, C. A. Czeisler, *Sleep Res.* **23**, 256 (1994)]. Thus, their disorder may be behaviorally self-induced via self-selected exposure to artificial light, rather than being the result of an aberrant intrinsic circadian period.
39. C. A. Czeisler, thesis, Stanford University (1978).
40. L. P. Morin, K. M. Fitzgerald, I. Zucker, *Science* **196**, 305 (1977); K.-I. Honma, S. Honma, T. Hiroshige, *Jpn. J. Physiol.* **35**, 643 (1985); D. M. Edgar and W. C. Dement, *Am. J. Physiol.* **261**, R928 (1991); P. J. Shiromani and D. Overstreet, *Biol. Psychiatry* **36**, 622 (1994); R. E. Kronauer, C. A. Czeisler, S. F. Pilato, M. C. Moore-Ede, *Am. J. Physiol.* **242**, R3 (1982); A. Gundel and M. B. Spencer, *Chronobiol. Int.* **9**, 148 (1992).
41. A. E. Hiddinga, D. G. M. Beersma, R. H. van den Hoofdakker, *J. Sleep Res.* **6**, 156 (1997).
42. J. Zulley and S. S. Campbell, *Hum. Neurobiol.* **4**, 123 (1985); N. Mrosovsky, *Nature* **319**, 536 (1986).
43. We reanalyzed the body temperature data recorded during the first week of our 28-hour forced desynchrony protocol (1 beat cycle) and the first 5 days of the 20-hour forced desynchrony protocol [also 1 beat cycle and identical to the duration of the experiment in (41)]. Estimation of circadian period on this shortened data series increased the variance of our period estimate by a factor of more than 13 in the case of the 28-hour protocol and by a factor of more than 6

- in the case of the 20-hour protocol, as would be predicted by our statistical model, in which the variance of the period estimate decreases as $1/T^3$ (37).
44. D. Minors et al., *Chronobiol. Int.* **13**, 179 (1996).
45. T. L. Kelly et al., *J. Biol. Rhythms* **14**, 190 (1999).
46. R. L. Sack, A. J. Levy, M. L. Blood, L. D. Keith, H. Nakagawa, *J. Clin. Endocrinol. Metab.* **75**, 127 (1992); D. N. Orth, G. M. Besser, P. H. King, W. E. Nicholson, *Clin. Endocrinol.* **10**, 603 (1979); S. W. Lockley et al., *J. Clin. Endocrinol. Metab.* **82**, 3763 (1997).
47. A. Eskin, in *Biochronometry*, M. Menaker, Ed. (National Academy of Sciences, Washington, DC, 1971), pp. 55–80.
48. F. C. Davis and N. Viswanathan, *Am. J. Physiol.* **275**, R960 (1998); J. F. Duffy, thesis, Northeastern University (1998).
49. M. A. Carskadon, S. E. Labyak, C. Acebo, R. Seifer, *Neurosci. Lett.* **260**, 129 (1999).
50. We thank the subject volunteers; research technicians; senior research technicians A. Fergus, K. Foote, G. Jayne, E. Martin, and A. Ward; S. Driscoll and the staff of the General Clinical Research Center (GCRC) of Brigham and Women's Hospital; subject recruiters J. Daley, J. Kao, D. Margolis, and R. McCarey; research assistants D. Chen and J. Whittemore Jr.; the Clinical Core Laboratory of the GCRC and Elias USA Inc. for hormonal assays; T. Ding for analytic software; W. Freitag for management of bioengineering systems; L. Rosenthal for illustrations; J. Zeitzer for comments; J. K. Wyatt for provision of comparative data; and G. H. Williams for overall support. Supported by the U.S. Public Health Service National Institute on Aging Award P01-AG09975 (C.A.C.), National Institute of General Medical Sciences Award R01-GM53559 (E.N.B.), and NASA Cooperative Agreement NCC9-58 with the National Space Biomedical Research Institute. The studies were performed in a GCRC supported by MO1-RR02635. This paper is dedicated to the memory of Professor Jürgen Aschoff (1913–1998), who pioneered the modern science of circadian biology and established the first continuously operational laboratory shielded from external time cues to study human circadian physiology.

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Arabidopsis Galactolipid Biosynthesis and Lipid Trafficking Mediated by DGD1

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The photosynthetic apparatus in plant cells is associated with membranes of the thylakoids within the chloroplast and is embedded into a highly specialized lipid matrix. Diacylglycerol galactolipids are common in thylakoid membranes but are excluded from all others. Isolation of the gene *DGD1*, encoding a galactosyltransferase-like protein, now provides insights into assembly of the thylakoid lipid matrix and subcellular lipid trafficking in *Arabidopsis thaliana*.

Of the four lipids associated with thylakoid membranes in plants only one is a phospholipid, the ubiquitous phosphatidylglycerol. The other three are nonphosphorous diacylglycerol glycolipids with one or two galactose moieties or a sulfonic acid derivative of glucose attached

to diacylglycerol (1). The galactolipids constitute the bulk (close to 80%) of the thylakoid lipid matrix and, within green plant parts, 70 to 80% of the lipids are associated with photosynthetic membranes. Most vegetables and fruits in human and animal diets are rich in galactolipids. Their breakdown products represent an important dietary source of galactose and polyunsaturated fatty acids (2).

Thylakoid membrane lipid biosynthesis in plants requires both carbohydrate and fatty acid metabolic pathways and is not restricted to chloroplasts, where galactolipids are found

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