± 2.4 g; and pellets-out, 100.7 ± 1.9 g. Parametrial fat pad weights: high fat, 5.6 ± 1.8 g; powder, 4.7 ± 0.5 g; pellets-in, 1.4 ± 0.2 g; and pellets-out, 0.8 ± 0.2 g.
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18. We thank M. I. Friedman and L. P. Morin who laid the conceptual groundwork for this research; J. D. Blaustein and G. J. DeVries for helpful comments on this manuscript; J. Alexander, R. Mergendahl, and M. H. Brown for technical assistance; and C. Bowden of McNeil Pharmaceuticals for the methyl palmoxirate. Supported by NIH research grants NS 10873-17 and AM 32976-06, National Institute of Mental Health Research Scientist Development Award MH 00321-09, and by NSF research grant BNS 8719361.

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Bright Light Induction of Strong (Type 0) Resetting of the Human Circadian Pacemaker

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The response of the human circadian pacemaker to light was measured in 45 resetting trials. Each trial consisted of an initial endogenous circadian phase assessment, a three-cycle stimulus which included 5 hours of bright light per cycle, and a final phase assessment. The stimulus induced strong (type 0) resetting, with responses highly dependent on the initial circadian phase of light exposure. The magnitude and direction of the phase shifts were modulated by the timing of exposure to ordinary room light, previously thought to be undetectable by the human pacemaker. The data indicate that the sensitivity of the human circadian pacemaker to light is far greater than previously recognized and have important implications for the therapeutic use of light in the management of disorders of circadian regulation.

THIRTY YEARS AGO, A PHASE RESPONSE curve (PRC) to light was first described (*1*), revealing the mechanism by which pacemakers driving circadian rhythms are synchronized (entrained) to the 24-hour day (*2*). Since then, PRC's to light have been described in nearly all eukaryotes studied except humans; it was believed that social contacts, rather than the light-dark cycle, synchronized the human circadian system to the 24-hour day (*3*). Subsequent studies demonstrated that the circadian system of normal subjects could be entrained by a 24-hour cycle of ordinary indoor room light and complete darkness (*4, 5*).

Evening exposure to bright light has been found to rapidly shift the phase of the endogenous component of the body temperature and cortisol cycles, even when the timing of the sleep-wake cycle was held constant (*6*). That experiment indicated that light could have a direct biological effect on the human circadian pacemaker, rather than an indirect synchronizing effect via its influence on the timing of sleep. We now report that the timing of light exposure has a critical effect on the magnitude and direction of the human circadian phase-resetting response to light. The human circadian pacemaker, which is more responsive to light than was previously postulated, can be reset to any desired phase by scheduled exposure to light for 2 to 3 days.

Typically, free-running activity rhythms of animals living for several weeks in constant darkness are interrupted by the presentation of brief light stimuli to evaluate the circadian resetting response of a pacemaker to light (*2*). However, in human subjects the free-running behavioral rest-activity cycle is an unreliable indicator of endogenous circadian phase and thus cannot be used to assess phase resetting (*7*). Hence, we have used the constant routine (CR) method to assess endogenous circadian phase (ECP), using the endogenous component of the body temperature cycle as a marker of the output of the human circadian pacemaker. Our CR procedure (*6*) is an extension and refinement of that of Mills (*8*), in which subjects are studied under constant environmental and behavioral conditions to unmask the endogenous component of the body temperature cycle by either eliminating, or distributing uniformly across the circadian cycle, physiologic responses to environmental and behavioral stimuli that can otherwise obscure it. Using the CR, we have found that repeated sequential estimates of the endogenous circadian phase at which the body temperature minimum (ECP_{min}) occurs (*9*) are highly correlated (Pearson's correlation coefficient, 0.998; $P < 0.001$), indicating that the CR is a reliable, reproducible procedure that has no measureable phase-shifting effect on the pacemaker. Therefore, we used the CR to assess the ECP_{min} both before and after the administration of a light stimulus in order to evaluate the phase-resetting effect of that stimulus (Fig. 1).

We applied a stimulus consisting of three cycles of exposure to a daily illuminance pattern that included bright light, ordinary indoor room light, and darkness (*10*) across the full range of initial circadian temperature phases (ϕ_i) (*11*). In order to determine the resetting response at these different phases, we began each resetting trial with an assessment of the initial pre-intervention ECP_{min} (t_1), exposed the subjects to the light stimulus, and then reassessed the final post-intervention ECP_{min} (t_2) (Fig. 1) (*11*). We conducted 45 resetting trials in 14 healthy young male subjects, aged 18 to 24 years (*12*), the data obtained represent a total of 420 subject-days of laboratory recording.

Exposure to our three-cycle light stimulus induced the largest phase shifts ($\Delta\phi > 8$ hours) when the light stimulus was centered around the initial ECP_{min} (t_1) (Fig. 2A). Since the ECP_{min} ordinarily occurs about 2 to 3 hours before the habitual wake time, centering the light stimulus around the ECP_{min} required inversion of the sleep-wake schedule (*13*); however, in 14 control trials in which the daily 5-hour episodes of bright light exposure were replaced with exposure either to room light (12 trials) or to darkness (2 trials), we found that such sleep-wake schedule inversion alone did not induce such large phase shifts (Fig. 2B) (*6*). This indicates that the observed phase shifts were induced primarily by the light exposure rather than by the displacement of sleep or activity.

To confirm that such light-induced phase shifts of the endogenous circadian temperature cycle accurately reflect phase shifts of the circadian pacemaker, we analyzed other established indicators of circadian rhythmicity before and after each of the resetting

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trials that resulted in a substantial ($\Delta\phi > 4$ hour) shift of the endogenous circadian temperature cycle (26 of the 45 trials). Even after substantial phase-advance or phase-delay shifts (Fig. 3), the average waveforms of circadian rhythms in plasma cortisol and urine output continued to bear the same phase relation to the endogenous circadian temperature cycle as they had during baseline CR's. This indicates that each of these rhythms was shifted by an equivalent amount and suggests that the cyclic bright light stimulus has shifted a master circadian pacemaker driving all three of these rhythms.

The phase shifts induced by the three-cycle stimulus used in these experiments were primarily dependent on the timing of exposure to bright light. We compared the results of our 23 resetting trials in which the subjects' daily exposure to bright light occurred midway through their daily 16-hour exposure to room light with trials in which the midpoint of the daily bright light exposure either preceded (11 trials) or followed (11 trials) that of the room light. We found that the timing of exposure to ordinary room light (~150 lux) compared to darkness (and sleep) can affect the magnitude and direction of phase shifts induced by bright light stimuli applied at peak light sensitivity (*13*) (Fig. 4). Therefore, we have incorporated the timing of exposure to both bright light and ordinary room light into the definition of the stimulus by calculating a brightness-weighted average of the midpoints of exposure to bright light and room light (*14*). We have used the phase of that brightness-weighted midpoint of the overall light exposure to denote the initial circadian phase (ϕ_i) at which we applied the light stimulus in all of the resetting trials.

The magnitude and direction of the phase shifts induced by the three-cycle light stimulus were dependent on the initial circadian phase at which the light exposure occurred (Fig. 5A). The largest phase shifts were observed when the stimulus occurred during the subjective night, reaching a maximum when the midpoint of the light stimulus was centered on the initial ECP_{min}. Advances to an earlier phase tended to occur when the light stimulus was centered late in the subjective night (after the initial ECP_{min}) while delays to a later phase occurred when the light stimulus was centered early in the subjective night (before the initial ECP_{min}) (Fig. 5A). Only small shifts were observed when the stimulus was centered within the subjective day. These results are consistent with the general properties of phase response curves to light described in all other species (*2*) and support our use of the temperature cycle measured during CR as an accurate assay of pacemaker phase.

To summarize our data, we have sought a mathematical representation, characterized by relatively few parameters, which approximates our understanding of essential circadian pacemaker properties. On the basis of our initial demonstration of a direct action of light on that pacemaker (*6*), Kronauer has recently proposed a mathematical model for

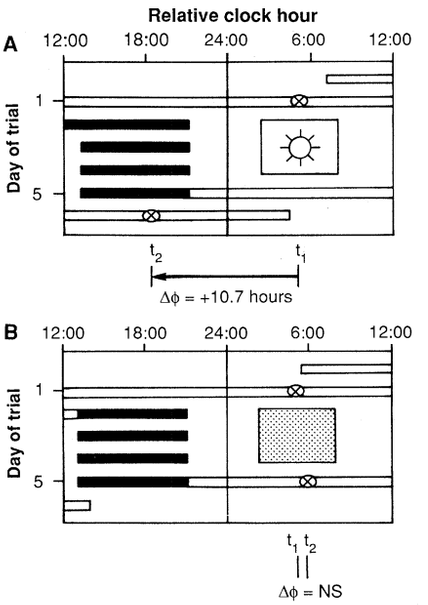

Fig. 2. Response of the circadian pacemaker to inversion of the sleep-wake schedule in an 18-year-old man (subject 720) exposed to either bright light (A) or darkness (B) centered around the initial ECP_{min}. (**A**) Rest-activity pattern plotted in a raster format, with successive days plotted beneath each other, illustrating a displacement of the sleep-wake cycle equivalent to that required for a 10.8 hour westward time-zone change. Symbols as in Fig. 1A, except that (i) constant routines are indicated by open bars; (ii) the time scale is defined relative to the subject's initial ECP_{min}, assigned a reference value of 05:00 (corresponding to the usual clock hour at which the ECP_{min} would occur in a healthy young man living in his home environment who sleeps from midnight to 8:00 a.m.); and (iii) the open box represents three cycles of daily exposure to a 5-hour episode of bright light (averaging 9843 lux) during the subject's scheduled daytime. After exposure to bright light centered around the subject's initial ECP_{min}, the subject's final ECP_{min} (t_2 = 18:18) was substantially phase advanced ($\Delta\phi$ = +10.7 hours) relative to the initial reference value (t_1 = 05:00). Using a Monte Carlo technique, our estimate of the standard error of each phase measurement is ±1.1 hours, or ±1.5 hours for the phase shift. (**B**) Control study of the same subject during a sleep-wake schedule displacement equivalent to that in (A). Stippled box represents three cycles of daily exposure to 5-hour episodes of darkness (< 0.02 lux) during the subject's scheduled daytime. Despite inversion of his sleep-wake schedule, the difference between the subject's final ECP_{min} (t_2 = 05:45) compared to its initial reference value (t_1 = 05:00) was not significant (NS).

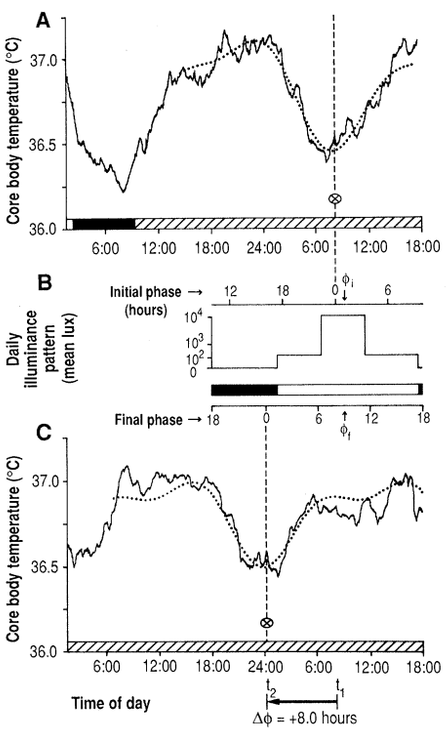

Fig. 1. Circadian phase resetting induced by light. (**A**) Before the initial phase assessment, the sleep (black bar) and wake (open bar) episodes of subject 706 (20-year-old male) were scheduled at their habitual times. The initial phase assessment consisted of a constant routine (CR) (hatched bar) in room light (averaging 150 lux) (*8*). Endogenous circadian phase (ECP) was assessed by fitting a two-harmonic regression model (dotted line) to the core body temperature data (solid line) from the CR (*9*). The resultant phase reference marker (ECP_{min}) is indicated by an encircled cross and a vertical dashed line; t_1 indicates the clock hour (8:10 a.m.) when the initial ECP_{min} occurred. (**B**) The light stimulus consisted of three cycles of exposure to a repeating daily pattern of illuminance: ordinary indoor room light (100 to 200 lux) throughout scheduled waketimes; bright light (7,000 to 12,000 lux, comparable in intensity to natural sunlight just after dawn) (*38*) for 5 hours per day, bracketed by 15 minutes of transitional illumination; and darkness (<0.02 lux) throughout scheduled sleep episodes. The average daily illuminance pattern (solid line) for subject 706 is plotted on a cube-root scale (*39*). The initial ECP_{min} (t_1) has been assigned a reference value of 0 on the initial phase scale. On that scale, ϕ_i indicates the initial circadian phase at which the midpoint of the overall light exposure occurred (1.0 hour after the initial ECP_{min}). On the final phase scale, the final ECP_{min} (t_2), observed after the intervention, has been assigned a reference value of 0; ϕ_f indicates the circadian phase of the light stimulus with respect to the final phase scale, where $\Delta\phi$ is the phase shift and $\phi_f = \phi_i + \Delta\phi$ (*11*). In this case, ϕ_f occurred 9.0 hours after the final ECP_{min}. (**C**) After the intervention, the final ECP_{min} (t_2) occurred at 00:11 a.m., 8.0 hours earlier than the initial ECP_{min} (t_1). This indicates that an 8-hour phase-advance shift ($\Delta\phi$ = +8.0 hours) was induced by the three-cycle intervention protocol.

the effect of light on the human circadian pacemaker, represented as a van der Pol oscillator (*15*). When stimuli are extended in time, as ours were, the intrinsic tendency of such a van der Pol oscillator to revert to its nominal amplitude would ordinarily act cumulatively to oppose stimulus-induced changes. However, if the oscillator's resiliency is weak [that is, its "stiffness" is low, as suggested in (*16*)], then even an extended-time stimulus can be approximated as an equivalent impulsive stimulus. Under such conditions, the asymptotic form achieved by this model (in the limit of weak stiffness) can be represented as:

$$\phi_f = \alpha - \frac{24}{2\pi}\tan^{-1}\left[\frac{\sin\left[\frac{2\pi}{24}(\phi_i - \phi_c)\right]}{a + (b-1)\cos\left[\frac{2\pi}{24}(\phi_i - \phi_c)\right]}\right] \quad (1)$$

where small deviations of the intrinsic circadian period from 24 hours are accommodated by the phase shifts, α and ϕ_c; and the parameter a represents the average influence of light and b represents a modulation of that influence as the cosine of its circadian phase of application. The sum $(a + b)$ represents the sensitivity to the stimulus when applied at $\phi_i = 0$ (that is, at ECP_{min}), while $(a - b)$ represents the sensitivity to the stimulus when applied at $\phi_i = 12$.

The data are well described by the parsimonious four-parameter representation (Fig. 5, solid lines), consistent with similar models of circadian phase resetting to single light stimuli acting impulsively on the oscillator (*17, 18*). The standard error of the model curve is comparable to that reported for *Drosophila pseudoobscura* (*17, 19*). The ratio $(a - b)/(a + b) = 0.22$ derived from our parameter estimation (Fig. 5C, legend) implies a substantial reduction of circadian sensitivity to light at $\phi_i = 12$ (relative clock hour 17:00), although more data are required in the band $9 < \phi_i < 21$ to estimate this ratio precisely. These data are consistent with reported circadian variations in retinal-visual sensitivity, which may be driven by an oscillator in the eye itself (*20*).

Our three-cycle stimulus has generated a phase transition curve (ϕ_f compared to ϕ_i, Fig. 5C) with an average slope of zero, comparable to Winfree's definition of strong "type 0" circadian phase resetting in response to single light pulses (*21*). This demonstration of type 0 resetting in human subjects, as anticipated by Winfree (*21*), implies that the stimulus has affected not only the pacemaker's phase, but also its amplitude of oscillation, consistent with our recent observations reported elsewhere (*22*).

Previously, the house sparrow (*Passer domesticus*) was the only vertebrate in which type 0 resetting by light had been reported, although Gander's data on the Polynesian rat (*Rattus exulans*) also support the possibility that mammals are capable of type 0 resetting in response to an extended (8- or 16-hour) light stimulus (*23*). While humans have been thought to be relatively insensitive to phase resetting by light (*3, 24*), our data indicate that the responsiveness of the human circadian pacemaker to light is within the wide range of sensitivity observed in

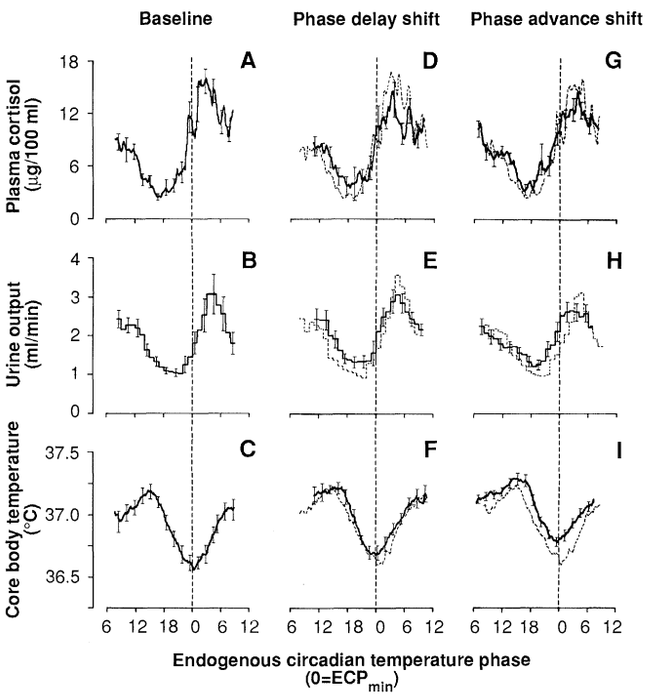

Fig. 3. Average waveforms of plasma cortisol, urine production, and core body temperature during baseline compared to waveforms after phase shifts of > 4 hours. (**A**, **B**, **C**) Average baseline waveforms (solid lines ± SEM) of each of these variables measured during the first constant routines (before intervention) of the 11 subjects who then underwent a phase shift of more than 4 hours. Data are aligned with respect to the baseline ECP_{min}, indicated by the vertical dashed line. (**D**, **E**, **F**) Average waveforms (solid lines ± SEM) of data recorded during constant routines after nine substantial phase-advance shifts in six subjects (mean $\Delta\phi$ ± SD = +7.0 ± 2.3 hours), aligned with respect to the final ECP_{min} (t_2). Plasma cortisol data are available for eight of the nine trials in these subjects. For direct comparison, these results are superimposed on average waveforms (broken lines) from the baseline constant routines, aligned with respect to the ECP_{min}, of those subjects who were subsequently phase-advance shifted; remaining symbols as in (A). (**G**, **H**, **I**) Average waveforms (solid lines ± SEM) from constant routines after 17 substantial phase-delay shifts in 11 subjects (mean $\Delta\phi$ ± SD = −6.9 ± 1.8 hours); remaining symbols as in (D to F). Plasma cortisol data are available for 14 of the 17 trials in these subjects. The average waveforms of circadian rhythms in adrenocortical and renal function maintained the same phase relation to the endogenous circadian temperature cycle during constant routines that followed substantial phase-advance or phase-delay shifts as they did during the baseline constant routines.

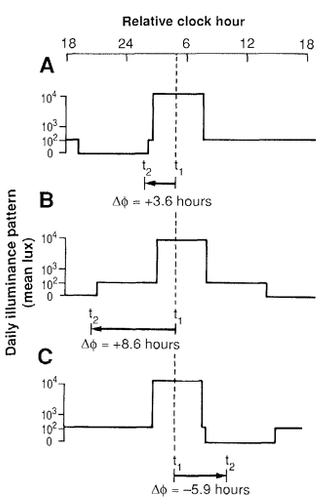

Fig. 4. The timing of exposure to room light can substantially modulate the phase-shifting effect of bright light when the midpoint of the bright light exposure, t_{BL}, occurs at the most light-sensitive phase. Daily illuminance patterns and resulting phase shifts are shown in three different trials of light exposure in a 22-year-old man (subject 713). In each of these trials, t_{BL} occurred at approximately the same initial ECP_{min} (t_1, indicated by a vertical dashed line), while the timing of exposure to room light [and therefore darkness or sleep (*13*)] was varied. In (**A**), exposure to room light occurred predominantly after the bright light exposure, whereas in (**C**), most of the exposure to room light occurred before the bright light exposure. In (**B**), the midpoint of the room light exposure (t_{RL}) was concurrent with that of the bright light exposure (t_{BL}). While t_{BL} occurred at a relative clock hour of 05:20 (± 15 minutes) in all three cases, the relative clock hours at which the midpoints of the overall light exposures occurred [t_L, which is a brightness-weighted average of t_{BL} and t_{RL}, as described in (*14*)] were 06:36, 05:34, and 03:43 for (A), (B), and (C), respectively. These t_L values correspond to initial circadian phases at which the stimuli occurred (ϕ_i) of 1.6, 0.6, and 22.7, respectively. These differences in ϕ_i were associated with marked differences in the magnitude and direction of the resetting response to the light stimulus [$\Delta\phi$ for (A) = +3.6 hours; for (B) = +8.6 hours; and for (C) = −5.9 hours], consistent with the results discussed below (in Fig. 5A).

lower organisms (*25*).

Furthermore, our results are not consistent with the suggestion that the human circadian timing system is unperturbed by exposure to ordinary indoor room light (*24, 26*); they are, however, consistent with earlier data on the effect of indoor room light on the synchronization of human glucocorticoid rhythms (*27*). Light probably exerts its primary action directly on the human circadian pacemaker via the monosynaptic retinohypothalamic tract (*2*), although pineal melatonin secretion may also exert some influence on the entrainment process (*28*).

Our results have implications for the treatment of circadian sleep disorders, such as the rapid time zone change (jet-lag) syndrome, delayed sleep phase syndrome (DSPS), hypernychthemeral (non-24-hour) sleep-wake syndrome, shift work dyssomnia and disrupted sleep in the elderly (*29*). These syndromes form a distinct class of sleep and arousal disorders and are characterized by a misalignment between the sleep-wake schedule and the endogenous circadian pacemaker, which is a major determinant of the timing of sleep and sleep stages in humans (*30, 31*). For example, recent laboratory studies simulating the phase shifts required for adjustment to transmeridian travel or rotating shift work show that even after 9 days, the body temperature rhythm is still not fully realigned with the new sleep-wake schedule following a 6-hour phase-advance shift (*32*). Our results indicate that with properly timed exposure to bright light, ordinary indoor room light, and darkness, physiologic adaptation to such a phase shift can be complete within 2 to 3 days. This may explain why transmeridian travelers who spend more time outdoors show quicker adaptation of their psychomotor performance rhythms to the new time zone than those who remain indoors (*33*), supporting the potential role of bright (outdoor) light exposure in the phase resetting process.

Preliminary clinical data suggest that exposure to bright light may be a rapid and practical treatment of both early morning awakening in the elderly and DSPS (*34*). Similarly, Lingjaerde *et al.* have successfully used morning bright light therapy to treat patients with an environmentally induced variant of DSPS, insomnia during the "dark period" in northern Norway (*35*). Exposure to bright light has also been reported to facilitate adaptation to a non-24-hour schedule (*36*), as may be required for aerospace exploration.

Finally, it has been found that seasonal (fall-winter) depression responds to phototherapy, although there are several hypotheses as to the mechanism of this effect (*22, 37*). Our characterization of a human phase response curve to bright light may prove to be important for understanding and effectively treating disorders of circadian regulation.

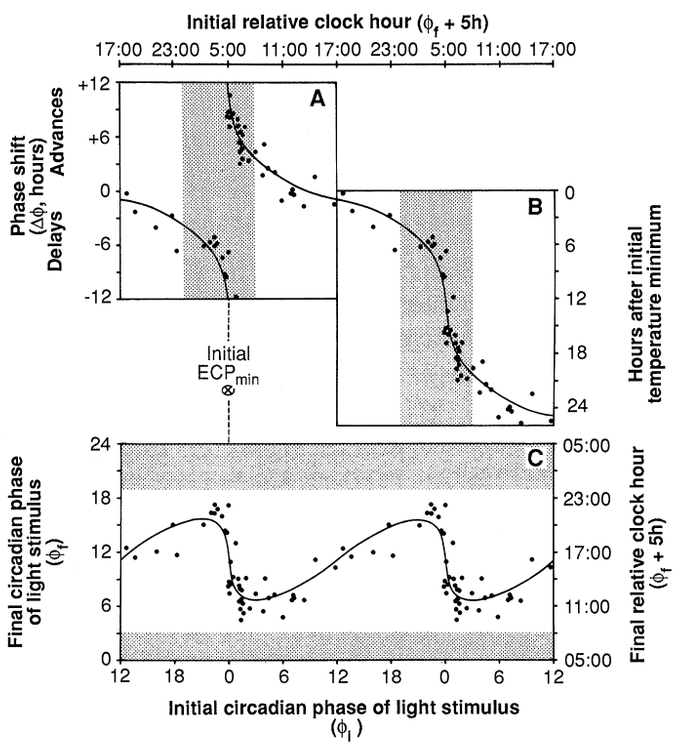

Fig. 5. Phase-dependent resetting responses of the human circadian timing system to light exposure. Resetting responses are plotted with respect to the initial circadian phase at which the light stimulus occurred (ϕ_i). In upper abscissa, initial ECP$_{min}$ is defined as 05:00; in lower abscissa, it is defined as 0. (**A**) Human phase response curve induced by exposure to the three-cycle light stimulus. The stippled area indicates the initial subjective night. Since the data are plotted in this standard format, there is an artifactual discontinuity, or "break point," in the curve during the subjective night when small differences in the initial circadian phase of the light stimulus appear to reverse the direction of the resulting phase shift. (**B**) Data from (A) are replotted with an ordinate which represents the number of hours between the ECP$_{min}$ after and before intervention, $t_2 - t_1$ (= $-\Delta\phi$). This projection reveals that there is not a true discontinuity in the phase response curve. (**C**) Human phase transition curve to the three-cycle light stimulus. Data are replotted in the format introduced by Winfree (*21*). Ordinate is ϕ_f, where $\phi_f = (\phi_i + \Delta\phi)$ mod 24. Stippled area represents the final subjective night. There is a 10-hour excluded zone during which ϕ_f never occurs, indicating that the light stimulus was strong enough to transform the phase at which the bright light exposure initially occurred (ϕ_i) into the subjective day, regardless of whether the initial phase of the stimulus was during the subjective day or subjective night. The mathematical representation in Eq. 1 was fit to the data in (C) with nonlinear least squares and then projected onto (A) and (B) (solid lines). Estimated parameters and their standard errors are: $\alpha = 11.06 \pm 0.3$; $\phi_c = 0.05 \pm 0.01$; $a = 0.70 \pm 0.1$; $b = 0.45 \pm 0.13$; covariance $(a, b) = -0.01$. Linear transformations of these variables yield the following estimates of the light sensitivity parameters: $(a + b) = 1.15 \pm 0.06$ (maximal effectiveness); $(a - b) = 0.25 \pm 0.23$ (minimal effectiveness). The standard error of the model [the root-mean-square deviations of the points (ϕ_f) from the model curve] is 1.99 hours. Based on the curve in (C), the maximal width of the 95 percent confidence interval for the mean of ϕ_f is ± 1.5 hours.

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7. Self-selected times of going to bed and rising during a free run do not occur at a consistent circadian phase in human subjects, even when the period of the rest-activity cycle is about the same as that of the pacemaker (*4, 30*). In humans, the behavioral rest-activity cycle is itself an oscillatory process with a labile period often substantially different from that of the pacemaker. Internal desynchronization between the free-running rest-activity cycle and the output of the circadian pacemaker, which occurs only in humans (*4, 30*), demonstrates that the rest-activity cycle is not a suitable marker of circadian phase in humans. In addition, many physiologic rhythms—including body temperature—are passively responsive to the rest-activity cycle, complicating attempts to accurately assess circadian phase resetting with such markers under free-running conditions. In fact, while Honma *et al.* have found that a single exposure to bright light can induce phase-advance shifts of the free-running rest-activity cycle in humans, they did not observe significant phase-delay shifts, regardless of the initial circadian phase of light exposure [K. Honma, S. Honma, T. Wada, *Experientia* **43**, 1205 (1987)]. We conclude that their failure to generate phase-resetting data consistent with data of other human studies (*6, 24*) or comparable to that of other species (*2*) is because the free-running rest-activity cycle in humans is not an accurate marker of the endogenous circadian pacemaker.
8. Mills *et al.* utilized 24-hour CR's beginning at 4:00 a.m., and allowed subjects to change posture occasionally [J. N. Mills, D. S. Minors, J. M. Waterhouse, *J. Physiol. (London)* **285**, 455 (1978); D. S. Minors and J. M. Waterhouse, *Chronobiol. Int.* **1**, 205 (1984)]. We have found the phase of the endogenous circadian temperature cycle minimum, the most reliable circadian phase marker, to be inadvertently obscured by the timing of the Mills procedure, since the masking effects of sleep on core body temperature persist for 3 to 5 hours after awakening occurs. We begin the CR at the regular

waketime, discard the first 5 hours of temperature data from the analysis, and extend the CR at least 8 hours after an unobscured temperature trough has occurred. Our subjects are restricted to absolute bedrest in a semi-recumbent posture, with wakefulness enforced by a research technician and verified by continuous polysomnographic recording [C. A. Czeisler *et al.*, *Sleep Res.* **14**, 295 (1985)] (*6*).

9. We fit the model to the temperature data using nonlinear least squares (E. N. Brown, thesis, Harvard University, 1987) and used an average of the minima from the single harmonic and composite waveforms of the model as the reference marker of endogenous circadian phase (ECP_{min}).
10. We have found that three or more cycles of bright light exposure (for 5 hours per cycle) induced phase shifts of a similiar magnitude, whereas fewer than three cycles of exposure or omission of bright light from the second and third cycles induced qualitatively different results, including substantial reduction of circadian amplitude (*22*). The endogenous circadian temperature amplitude observed after substantial (> 4-hour) phase shifts induced by the three-cycle stimulus was about 10 to 15 percent below normal. Pilot studies indicate that an additional (4th) cycle of exposure normalized amplitude but did not substantially change the final circadian phase (ϕ_f), suggesting that the transients which normally precede attainment of a new steady-state phase after an intervention were largely complete prior to the final phase assessment.
11. The initial circadian phase of the light stimulus (ϕ_i) is given as $(t_L - t_1)$ mod 24, where t_L is the time of the brightness-weighted average midpoint of the light stimulus (*14*). The final circadian phase of the light stimulus (ϕ_f) is defined as $(t_L - t_2)$ mod 24 (see Fig. 1). The phase shift ($\Delta\phi$) is calculated as: $(t_1 - t_2)$ mod 24, where t_1 is the time of the initial ECP_{min} and t_2 is the time of the final ECP_{min}. By convention, phase delays ($\Delta\phi < 0$) represent shifts to a later hour on the reference time scale and phase advances ($\Delta\phi > 0$) represent shifts to an earlier hour.
12. Subjects had no evidence of medical, psychiatric, or sleep disorders as determined by clinical history, physical examination, chest radiograph, electrocardiogram, clinical laboratory screening tests, and psychological screening questionnaire (Minnesota Multiphasic Personality Inventory). Urinary screening verified that all subjects were drug-free at the time of study. Informed consent was obtained from all subjects, who were studied for one to ten trials, depending on availability. In the five subjects with at least four sequential resetting trials, phase resetting responses across a range of initial phases were all close to the estimated response curve derived from the entire group, suggesting that interindividual differences were small. Moreover, when stimuli were repeated at about the same initial circadian phase within a subject, resetting responses were similar, suggesting that intraindividual differences were small.
13. Since the subjects slept during scheduled dark episodes, the apparent effect of room light could be due in part to an effect of the rest-activity cycle on the pacemaker, as recently described in animals [N. Mrosovsky and P. A. Salmon, *Nature* **330**, 372 (1987); O. Van Reeth and F. W. Turek, *ibid.* **339**, 49 (1989)]. However, free-running endocrine rhythms with an intrinsic period as low as 24.35 hours have been reported in blind subjects whose rest-activity cycles were constrained to 24 hours [L. E. M. Miles, D. M. Raynal, M. A. Wilson, *Science* **198**, 421 (1977); D. N. Orth, G. M. Besser, P. H. King, W. E. Nicholson, *Clin. Endocrinol.* **10**, 603 (1979); R. L. Sack, T. M. Hoban, A. J. Lewy, *Sleep Res.* **16**, 636 (1987)], indicating a range of entrainment of those rhythms to the rest-activity cycle alone of less than 0.35 hour. The range of entrainment for such rhythms is three times larger (about 1.2 hours) in normally sighted subjects whose rest-activity cycle is similarly constrained to 24 hours, but who are also exposed to a concurrent cycle of ordinary indoor room light and darkness [J. E. Fookson *et al.*, *Sleep Res.* **13**, 220 (1984)]. We therefore estimate that the rest-activity cycle contributes ≤ 30 percent of the total entraining effect of the imposed schedule of ordinary indoor room light and darkness or sleep.
14. Small displacements of ϕ_i due to changes in the timing of room light can only have a substantive effect when ϕ_i is at the steepest point on the response curve (at the ECP_{min}, as in Fig. 4). Using the formula: $t_L = (k)t_{BL} + (1 - k)t_{RL}$, where t_L is the brightness-weighted midpoint of the overall light pattern, t_{BL} is the midpoint of bright light, and t_{RL} is the midpoint of room light. Our working estimate of the weighting ratio $[(k/(1 - k)]$ is 2.7. Other experiments are required for a precise estimate. In any event, the phase-resetting curve derived from our data with $\phi_i = (t_{BL} - t_1)$ mod 24 is qualitatively the same as that from $\phi_i = (t_L - t_1)$, since the mean difference between the actual value of t_{BL} and that calculated for t_L was only 0.66 hour (maximum 1.5 hours) for the resetting trials reported here.
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19. Both ϕ_i and ϕ_f were observed with error, arising from uncertainty in their estimation, inter- and intrasubject variability of responsiveness, and potentially from incomplete transients in subjects who underwent sequential resetting trials. To minimize these sources of error, only trials in which the endogenous circadian temperature amplitude exceeded 0.15°C during each initial CR were included. The model goodness of fit (Fig. 5C) suggests that these sources of error were small relative to the phase-shifting effects of the light; resetting responses observed in subjects' first resetting trials were indistinguishable from subsequent responses. Analysis of nonlinear errors in variables, along with further experiments, are necessary to quantify further these sources of error.
20. Endogenous circadian rhythms have been reported in diverse functions of the visual system in various species [J. W. Jacklet, *Science* **164**, 562 (1969); J. N. Lythgol and J. Shand, *Invest. Opthalmol. Vis. Sci.* **24**, 1203 (1983); J. Brandenburg, A. C. Bobbert, F. Eggelmeyer, *Behav. Brain Res.* **7**, 113 (1983); M. M. LaVail, *Science* **194**, 1071 (1976); A. Wirz-Justice, M. Da Prada, C. Reme, *Neurosci. Let.* **45**, 21 (1984)], including human visual sensitivity [R. Knoerchen and G. Hildebrandt, *J. Interdisc. Cycle Res.* **7**, 51 (1976)]. While considerable evidence indicates that in some species the eyes contain a circadian oscillator [G. D. Block and S. F. Wallace, *Science* **217**, 155 (1982); T. L. Page, *Science* **216**, 73 (1982); M. Terman and J. Terman, *Ann. N.Y. Acad. Sci.* **453**, 147 (1985)], mammalian studies suggest that the oscillator is synchronized to the light-dark cycle via its neural connections to the central nervous system, not by the direct exposure of the eye to light [P. S. Teirstein, A. I. Goldman, P. J. O'Brien, *Invest. Ophthalmol. Vis. Sci.* **19**, 1268 (1980)].
21. The peak-to-peak amplitude (PP) and the distance between peaks (W) of the phase transition curve (Fig. 5C) were approximately 11 hours and 5 hours, respectively, as defined by Winfree (*17*). These values are comparable to those derived from the phase transition curve to 2 hours of exposure to 8000 lux in the mosquito *Culex pipiens quinquefasciatus* [E. L. Peterson, *J. Theor. Biol.* **84**, 281 (1980)]; in both cases, the sawtooth shape of the curves indicates that the strength of the stimulus was just sufficient for strong type 0 resetting [A. T. Winfree, in *Biochronometry*, M. Menaker, Ed. (National Academy of Sciences, Washington, DC, 1969), pp. 81–109; *The Geometry of Biological Time* (Springer-Verlag, New York, 1980); A. T. Winfree, *The Timing of Biological Clocks* (Scientific American Books, New York, 1987)]. Since three cycles of light exposure was just sufficient to induce type 0 resetting in our subjects, it is unlikely that strong type 0 resetting can be achieved with a single exposure to a tolerable intensity of light in normal young men.
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25. The belief that lower organisms are much more sensitive to light than vertebrates may stem from studies of pupal emergence in *Drosophila pseudoobscura* [C. S. Pittendrigh, *Cold Spring Harbor Symp. Quant. Biol.* **25**, 159 (1960)]. Those pupae are so sensitive to light that 55 seconds of dim blue light (1 W/m^2) results in strong type 0 resetting of the circadian pacemaker gating that critically timed event (*17*). However, adult flies of the same species require 12 hours of white light at 7000 lux to achieve comparable type 0 resetting of their circadian activity [W. Engelmann and J. Mack, *J. Comp. Physiol.* **127**, 229 (1978)]. The cockroach *Leucophaea maderae* requires 80,000 lux for 12 hours to achieve strong type 0 resetting of its locomotor activity rhythm [G. Wiedenmann, *Z. Naturforsch.* **32**, 464 (1977)].
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38. The bright light was provided by a bank of either cool-white Econ-o-watt (North American Philips Lighting) or Vitalite wide-spectrum (Duro-test) fluorescent lamps, applied in addition to ordinary indoor room lighting. The phase shifts induced by these different light sources were equivalent for a given level of illuminance (in lux). During bright light exposure, each subject was seated facing a vertical bank of fluorescent lamps and instructed to look directly at them for 5 of every 10 minutes. Illuminance was recorded at 5-minute intervals (*6*). Each subject's daily exposure to ultraviolet (UV) light during the trials was well within safety guidelines [*Documentation of the Threshold Limit Values and Biological Exposure Indices, Fifth Edition* (American Conference of Governmental Industrial Hygienists, Inc., Cincinnati, 1986); D. H. Sliney, *Am. J. Op-*

tom. Physiol. Opt. 60, 278 (1983); National Institute for Occupational Safety and Health, *Criteria for a Recommended Standard, Occupational Exposure to Ultraviolet Radiation* (National Technical Information Service, Rockville, MD, 1972, Government Publication No. PB-214 268)]. Our subjects now wear UV-excluding clear Ultra-spec 2000 safety glasses (Uvex Winter Optical, Inc., Smithfield, RI) during bright light exposure, confirming that UV light is not responsible for the phase resetting observed.

39. Kronauer (*15*) suggests that the illuminance of light (as measured in lux) is related nonlinearly to its biological influence on the endogenous circadian pacemaker, and that the experimentally determined [S. S. Stevens, *Science* **133**, 80 (1961)] relation between illuminance (I) and perceived brightness (B), $B = CI^{1/3}$, might apply here. This has proved effective in the model simulation of laboratory bright light protocols [R. E. Kronauer and J. V. Frangioni, *Sleep Res.* **16**, 622 (1987)].

40. We thank the subject volunteers; the student research technicians; A. E. Ward, M. J. Matule, A. M. Sinz and M. P. Johnson for direct supervision of the studies; K. Whitmarsh and J. W. Jewett for subject recruitment; J. L. Anderson and S. A. Amira for psychological evaluations; S. Rogacz, J. Steinberg, and R. Mortensen for clinical evaluations; J. Swain, R. Salvador, and M. McCullough for the metabolic diets; L. Thorington for advice on designing the light banks; B. Potter and J. Norwood for hormonal assays; A. E. Ward, D. Sieburth and S. Lawson for the illustrations; M. Dumont, W. O. Freitag, J. Miner and S. H. Strogatz for suggestions on the manuscript; and G. H. Williams for his overall support of this work. Supported in part by grants NIA 1-RO1-AG06072, NIH DRR GCRC 5-M01-RR00888, NIH DRR BRSG 2-S07-RR-05950; by NIDDK training grant 5-T32-OK-07529 (E.N.B.); and by the Center for Design of Industrial Schedules. C.A.C. is a Sandoz Scholar in Medicine.

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Water-Inserted α-Helical Segments Implicate Reverse Turns as Folding Intermediates

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Information relevant to the folding and unfolding of α helices has been extracted from an analysis of protein structures. The α helices in protein crystal structures have been found to be hydrated, either externally by a water molecule hydrogen bonding to the backbone carbonyl oxygen atom, or internally by inserting into the helix hydrogen bond and forming a hydrogen-bonded bridge between the backbone carbonyl oxygen and the amide nitrogen atoms. The water-inserted α-helical segments display a variety of reverse-turn conformations, such as type III, type II, type I, and opened out, that can be considered as folding intermediates that are trapped in the folding-unfolding process of α helices. Since the α helix, most turns, and the extended β strand occupy contiguous regions in the conformational space of φ, ψ dihedral angles, a plausible pathway can be proposed for the folding-unfolding process of α helices in aqueous solution.

ALL OF THE INFORMATION required for the tertiary folding of a protein is contained in its primary sequence (*1*). However, protein folding is a fast process that makes characterization of the folding intermediates difficult (*2, 3*). We have examined native protein structures for hints of what may have happened during their folding. We have found that a water molecule binds to an α helix, either externally to the backbone carbonyl O atom (Fig. 1A) or internally by prying open the helix hydrogen bond and lodging between the backbone carbonyl O atom and the amide group (Fig. 1B). The local conformations of the internally solvated α helices adopt an ensemble of classical reverse-turn conformations (*4*), types I, II, III, and open turns including the 3_{10} helix, that could represent trapped intermediates in the unfolding or folding of α helices. These intermediate structures occupy either common regions or are proximal to each other in the Ramachandran conformational space (*5*), and allow us to propose plausible folding pathways of α helices.

The impetus for this work began with our observations on the modes of hydration of α helices in troponin C (*6*), in which the exposed helix "handle" was surrounded by water molecules that hydrogen-bonded to the backbone carbonyl O atoms and the first turn of the B helix was disrupted by the insertion of water molecules into the helix hydrogen bonds. A similar binding of water molecules was simultaneously found in the structure of a small molecule, a synthetic oligopeptide analog containing α-aminoisobutyric acid residues (*7*). We surmised that these hydration schemes represent steps in the unfolding of α helices (*6*). The water-inserted segments displayed reverse-turn conformations, which suggested that the turn could be an incompletely folded helical segment that was trapped during the folding-unfolding of the α helices. Additional evidence for this arose when we noted the interchangeable occurrence of the helical segments and reverse turns in structures of the same protein from different sources: for example, phospholipase A2 residues 58 to 62, bovine (1BP2) (helical) versus porcine (1P2P) (turn); lysozyme 112 to 115, human (1LZ1) versus chicken (1LZT); acid protease residues 128 to 130, 161 to 163, and 176 to 178, penicillium (2APP) versus rhizopus (2APR). In the similarly folded chymotrypsin family of serine proteases, the helix content and the number of water-inserted segments differ, 2ALP (one helix), 3EST (two helices and one inserted segment, 233 to 237), 3RP2 (three helices and one inserted segment, 233 to 237), 2PTN (three helices and two inserted segments, 172 to 176 and 233 to 237), 4CHA (three helices and one inserted segment, 232 to 236), and 1TON (four helices and three inserted segments, 173 to 177, 232 to 236, and 233 to 237), again revealing the interchangeability of helical and nonhelical segments. In troponin C, there are four homologous calcium-binding helix-loop-helix motifs, namely, A, B; C, D; E, F; and G, H, but only the B helix contained inserted waters, whereas the other helices did not (*6*).

We collected hydrated α-helical segments from 35 protein structures (Table 1) that have been refined at a resolution of 1.9 Å or better from the Brookhaven Protein Data Bank (*8*). Only one structure from a family of proteins was included in our analysis. In serine proteases, one each from chymotrypsin and subtilisin families was used. We used the criterion that the hydrated "helical" segment should contain at least two residues in common with a helix, inclusive of the terminal residue, and also be compatible with retention of the adjacent helical conformation as visualized on a PS300 system using FRODO. Hence, we used $N - 3$ and $C + 3$ as helix boundaries, where N and C are the positions in the sequence of the helix terminal tersidues, as found in the Brookha-

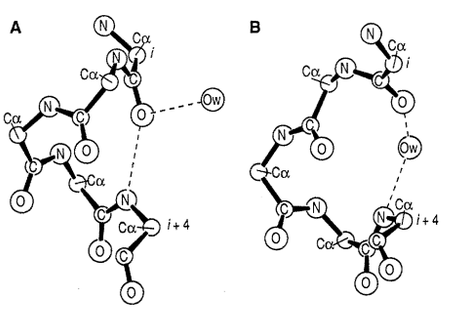

Fig. 1. The local pentapeptide segment of an α helix (**A**) with an externally bound water to the backbone carbonyl O atom, and (**B**) with an internally bound water bridging the backbone carbonyl O_i of the ith residue and the amide nitrogen atom N_{i+4}.

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