

Circadian Clock Resetting by Sleep Deprivation without Exercise in Syrian Hamsters: Dark Pulses Revisited

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Abstract Circadian rhythms in Syrian hamsters can be phase shifted by procedures that stimulate wheel running ("exercise") in the mid-subjective day (the hamster's usual sleep period). The authors recently demonstrated that keeping hamsters awake by gentle handling, without continuous running, is sufficient to mimic this effect. Here, the authors assessed whether wakefulness, independent of wheel running, also mediates phase shifts to dark pulses during the mid-subjective day in hamsters free-running in constant light (LL). With running wheels locked during a 3 h dark pulse on day 3 of LL, hamsters ($N = 16$) averaged only 43 ± 15 min of spontaneous wake time and phase shifted only 24 ± 43 min. When wheels were open during a dark pulse, two hamsters remained awake, ran continuously, and showed phase advance shifts of 7.3 h and 8.7 h, respectively, whereas the other hamsters were awake < 60 min and shifted only 45 ± 38 min. No animals stayed awake for 3 h without running. Additional time in LL (10 and 20 days) did not potentiate the waking or phase shift response to dark pulses. When all hamsters were sleep deprived with wheels locked during a dark pulse, phase advance shifts averaged 261 ± 110 min and ranged up to 7.3 h. These shifts are large compared to those previously observed in response to the 3 h sleep deprivation procedure. Additional tests revealed that this potentiated shift response is dependent on LL prior to sleep deprivation but not LL after sleep deprivation. A final sleep deprivation test showed that a small part of the potentiation may be due to suppression of spontaneous wheel running by LL. These results indicate that some correlate of waking, other than continuous running, mediates the phase-shifting effect of dark pulses in the mid-subjective day. The mechanism by which LL potentiates shifting remains to be determined. The lack of effect of subsequent LL on the magnitude of shifts to sleep deprivation in the dark suggests that LL reduces responsivity to light by processes that take > 3 h of dark to reverse.

Key words sleep deprivation, wheel running, entrainment, circadian rhythm, nonphotic zeitgeber

Circadian rhythms in nocturnal and diurnal mammals can be phase shifted or entrained by procedures

that stimulate locomotor activity. This phenomenon has been most thoroughly studied in the Syrian ham-

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ster. Hamsters can be induced to run in the middle of their subjective day (the rest phase in nocturnal species) by confinement to a novel running wheel (Reebs and Mrosovsky, 1989). The distribution of running responses is typically bimodal; hamsters either run a lot (virtually continuously) or very little. Those that run continuously (the majority) typically produce 4000 to 10,000 wheel revolutions in 3 h (about 2.5 to 5 km) and exhibit substantial phase advance shifts, averaging up to 3.5 h (e.g., Janik and Mrosovsky, 1993; Mistlberger et al., 1996; Bobrzynska and Mrosovsky, 1998). Hamsters that run little typically fail to shift. Phase shifting of this kind is thus commonly referred to in the literature as "activity dependent." The strong association between running and shifting suggests that the clock is reset by some neural or endocrine correlate of continuous, high-intensity locomotor activity. This interpretation has prompted the study of nighttime exercise as a phase-resetting stimulus in humans (e.g., Van Reeth et al., 1994; Buxton et al., 1997).

Recently, we demonstrated that phase shifts induced by stimulated running in hamsters can be mimicked by 3 h of waking without running, enforced in the middle of the subjective day by the sleep deprivation method of gentle handling (Antle and Mistlberger, 2000). Locomotor activity during this procedure is minimal (averaging ~0.08 km/3 h). These results indicate that continuous running is not necessary for maximal clock resetting. Presumably, among hamsters studied in the novel wheel confinement paradigm, "runners" shift because they are awake continuously and "nonrunners" fail to shift because they sleep.

If this hypothesis is correct, then we should see a relationship between spontaneous waking and phase resetting in other paradigms that have provided support for "activity-dependent" resetting of circadian rhythms. However, few studies have collected data on behavioral state independent of wheel running. One study reported a significant positive association between the rate of reentrainment to an 8-h advance of the LD cycle and the behavioral state of hamsters noted at hourly intervals following lights-off on the day of the advance; hamsters that were awake early in the first dark period (formerly the middle of the day) reentrained more rapidly than hamsters observed to be asleep (Honrado and Mrosovsky, 1989). Other studies have demonstrated that brief handling to administer saline by intraperitoneal injection late in the subjective day (but not at other phases) can induce

small phase advance shifts that are related to cortisol secretion but not to locomotor activity (Hastings et al., 1992).

Here, we report on the role of waking, with and without running, in the induction of phase shifts by exposure to dark pulses. Darkness in the mid-subjective day can trigger wheel running in hamsters maintained in constant light (LL) (e.g., Aschoff and von Goetz, 1988). Dark pulses of 2 to 6 h duration induce phase shifts similar in magnitude and timing to those induced by running in a novel wheel (e.g., Boulos and Rusak, 1982). If running during the dark pulse is prevented by disabling the wheel or confining the hamsters to a nest box or restraint tube, phase shifts are either attenuated or absent (Reebs et al., 1989; Van Reeth and Turek, 1989). These results have been interpreted as evidence that locomotor activity mediates clock resetting in response to darkness. But, without wheel access, hamsters may sleep through much of the dark pulse. Consistent with this idea, it was noted in a pilot study that hamsters prevented from wheel running by confinement to a nest box during a dark pulse made little noise, suggesting that they were inactive and possibly asleep (Reebs et al., 1989). To examine this issue more closely, we quantified behavioral state and phase shifts associated with dark pulses, with and without concomitant sleep deprivation by gentle handling. In additional experiments, we examined the influence of prior and subsequent lighting on the magnitude of phase shifts associated with darkness and enforced waking.

GENERAL METHODS

Subjects and Apparatus

Young adult male Syrian hamsters ($N = 16$) were received at 75 g (Charles River, Quebec, Canada) and housed individually in plastic cages ($45 \times 25 \times 20$ cm) equipped with running wheels (17 cm diameter) and wire floors. Wheel running was detected by microswitches monitored continuously by microcomputer. Activity data were summed and saved to disk at 10-min intervals. The cages were maintained in a ventilated isolation cabinet with incandescent lighting (14:10 LD). Light intensity was 0 lux in the dark and averaged 16 ± 13 lux across cages during lights-on, measured from the center of each cage, with the light meter facing the nearest light source. The maximum lux values obtainable averaged 96 lux across cages.

Table 1. Group mean phase shifts to treatment conditions, listed in chronological order (tests 1 and 2, and 4 and 5 were counterbalanced).

Test	Condition	Phase Shift (min) SD
1.	Dark pulse with wheel locked, LL day 3	24 ± 43
2.	Dark pulse with wheel open, LL day 3	103 ± 158
3a.	LL to DD step transition with wheel locked, LL day 3	39 ± 45
3b.	LL to DD step transition with wheel open, LL day 3	94 ± 92
4.	Dark pulse with wheel locked, LL day 10 or 20	16 ± 52
5.	Dark pulse with wheel open, LL day 10 or 20	44 ± 78
6.	Dark pulse with sleep deprivation, LL day 3	261 ± 110
7.	LL to DD step transition with sleep deprivation, LL day 3	234 ± 53
8.	Sleep deprivation, DD day 3	152 ± 65
9.	Sleep deprivation, DD day 3, wheel locked DD days 1-3	162 ± 64

NOTE: There was a significant main effect of treatment ($F_{9,124} = 15.3$, $p < 0.0001$). Assumptions of normality and homogeneity of variance were met. Specific post hoc comparisons were conducted on n s of 12 to 16 (see text for results of specific pairwise comparisons). LL = constant light, DD = constant darkness.

Procedures

The hamsters were subjected to nine tests (referred to below as tests 1 to 9), separated by at least 7 days in LD 14:10, over a total recording period of 190 days. The order and counterbalancing of these tests are provided in Table 1. Dark pulses with or without sleep deprivation were initiated at projected circadian time 6 (CT 6, where, by convention, CT 12 is defined as the onset of the daily active period) after 2 days in constant dark (DD) or 2, 10, or 20 days in LL. Two days was used as the standard in all but two tests to ensure that circadian phase did not diverge substantially across animals before the manipulations. During dark pulses without sleep deprivation, hamsters were observed at 1-min intervals, and behavioral state was scored as wake (head up, eyes open) or sleep (head down, curled sleep posture, eyes closed if in view). Lighting was dim red (<1 lux), with observation aided by an infrared viewer. During dark pulses with sleep deprivation, hamster cages were removed from the cabinet and placed on the floor, in semicircles of three or four, each manned by one investigator. Cage tops were removed, and running wheels were locked. Hamsters were prevented from leaving the cages but not from moving within the cage. Hamsters that attempted to adopt a sleep posture were stimulated with a light puff of air or touch of the whiskers or flank. An infrared videotape was made to confirm

standardization of technique with prior work (Antle and Mistlberger, 2000). At the end of the 3-h procedure, the cages were returned to the isolation cabinet and the animals were left undisturbed for 4 or more days in LL or DD.

Data Analysis

Phase shifts were quantified by comparing the time of spontaneous activity onset (CT 12) on the second day after the procedure with activity onset on the day prior to the procedure. Extensive prior work indicates that phase shifts induced by stimulated running or sleep deprivation are fully expressed by that time (e.g., Antle and Mistlberger, 2000). Activity onsets were defined by computer algorithm as the first 10 min time bin in which wheel revolutions exceeded 25, following an interval of 4 or more hours in which they did not exceed this level. Differences between conditions were evaluated statistically by repeated-measures analysis of variance (SigmaStat, SPSS Sciences, Inc., Chicago, IL, USA). Post hoc multiple pairwise comparisons were made using the method of Student-Newman-Keuls ($\alpha = 0.05$). Relationships between phase shifts, wheel running, and wake time were evaluated using Pearson bivariate correlations. Results are expressed as mean $\pm$ SD.

RESULTS AND DISCUSSION

Tests 1 and 2: Spontaneous Waking, Running, and Shifting Effects of Dark Pulses

We examined first whether variability in the phase-shifting response to 3 h dark pulses in LL was related to individual differences in spontaneous waking and wheel running during the dark pulse. Each hamster was tested twice, once with the wheel locked and once with the wheel open during the dark pulse (tests 1 and 2, counterbalanced for order).

During 3 h dark pulses in which the wheels were locked, the hamsters averaged 43 ± 15 min (range: 26 to 72 min) of spontaneous wake time. Phase advance shifts measured on the second day after the procedure averaged 24 ± 43 min (range: -70 to 70 min). There was no significant association between shift size and spontaneous wake time ($r = -0.14$, $p > 0.1$).

During 3 h dark pulses in which the wheels were open, the hamsters averaged 69 ± 45 min of spontaneous wake time (range: 24 to 180 min) and 103 ± 158 min

phase advance shifts (range: -20 to 520 min). Spontaneous wake time and total wheel revolutions were strongly related ($r = 0.95$), as was phase shift magnitude with wake time ($r = 0.91$) and with wheel revolutions ($r = 0.83$). These correlations largely reflect a bimodal distribution of acute behavioral responses to darkness (Fig. 1); two hamsters were awake for 161 and 180 min, respectively, during which they produced 4493 and 5423 wheel revolutions, respectively. Phase advance shifts in these two cases were exceptional (440 and 520 min, respectively) (Fig. 2) in comparison with the remaining animals (45 ± 38 min) and with their own shifts in the wheel-locked condition (wake times of 66 and 44 min, respectively, and shifts of 0 and 50 min, respectively) (Fig. 2).

Tests 3a and 3b: Role of Lights-On after a Dark Pulse: Step Changes in Lighting

Tests 1 and 2 demonstrated that phase shifts to dark pulses are minimal when hamsters do not have access to a running wheel or do not run in the wheel. Even with wheel access, surprisingly few animals stayed spontaneously awake throughout the dark pulses, and no significant correlation with shift size was detected among nonrunners. Previous studies have demonstrated that light can inhibit phase shifts to nonphotic stimuli in the mid-subjective day (e.g., wheel running, Mrosovsky, 1991; sleep deprivation, Antle and Mistleberger, 2000; ($\pm$)8-hydroxydipropylaminotetralin [8-OH DPAT], Ehlen et al., 2001). Transient (~60 min) awakening by darkness might constitute a weak behavioral zeitgeber vulnerable to inhibition by subsequent lights-on. If so, then phase shifts might be larger if the lights remained off, which might unmask a relationship between shift size and the amount of wake time in the first 3 h of darkness. Photic inhibition could explain why phase shifts observed in response to dark pulses were much smaller than shifts observed following transitions from LL to DD initiated at the same phase (Albers, 1986).

To test this prediction, the hamsters were exposed to LL for 2 days, followed by DD for 4 days, beginning at CT 6. The LL to DD step change is identical to the dark pulse procedure, until the point at which LL would normally resume. Running wheels were locked in half of the group (test 3b).

The results of these manipulations were virtually identical to the results of tests 1 and 2. Hamsters with wheel access during the first 3 h of dark shifted 94

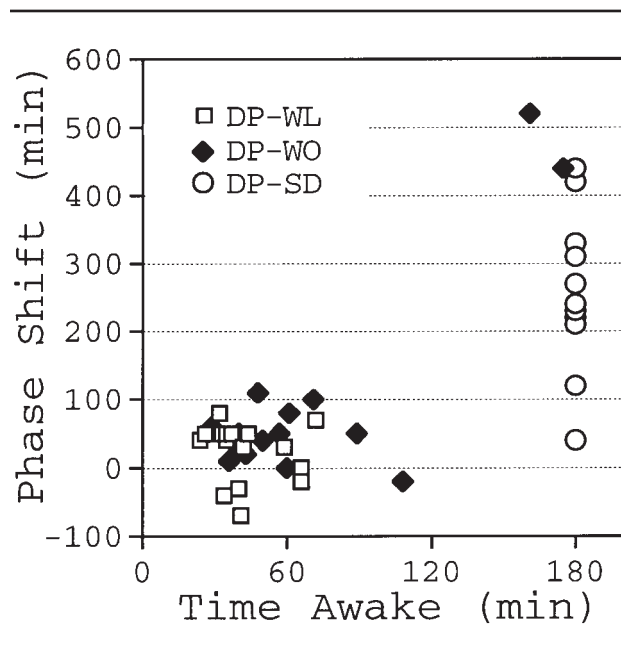


Figure 1. Phase shifts in minutes plotted against spontaneous wake time during 3 h dark pulses with wheels locked (DP-WL, squares) or wheels open (DP-WO, filled diamonds) and against total wake time during 3 h of sleep deprivation (DP-SD, circles).

± 92 min, whereas those with wheels locked shifted 39 ± 45 min (e.g., Fig. 2). The same set of animals ($n = 14$ [data missing in 2 cases]) shifted 89 ± 176 min and 16 ± 42 min, respectively, in the dark pulse conditions (tests 1 and 2; differences between dark pulse and step change conditions not significant within these groups). In the wheel-open condition, the group mean was heavily influenced by a single hamster that ran 6710 wheel revolutions and shifted 310 min (Fig. 2). The remaining hamsters ran 1020 or fewer revolutions and shifted 63 ± 32 min. These results indicate that lights-on after a dark pulse is not responsible for the lack of phase shifting in hamsters that exhibit less than ~60 min of spontaneous waking combined with less than ~1000 wheel revolutions in 3 h.

Tests 4 and 5: Effect of Time in LL on Phase Shifts to Dark Pulses

In tests 1 to 3, dark was initiated on day 3 of LL. Previous dark pulse and LL to DD step transition studies used 10 days or more of LL prior to tests and reported much larger mean shifts (Boulos and Rusak, 1982; Albers, 1986). One possibility is that extended time in LL makes dark pulses in the mid-subjective day a more potent stimulus for waking. To examine this, the hamsters were reentrained to LD and then exposed to LL for 30 days. On days 10 and 20, the hamsters were

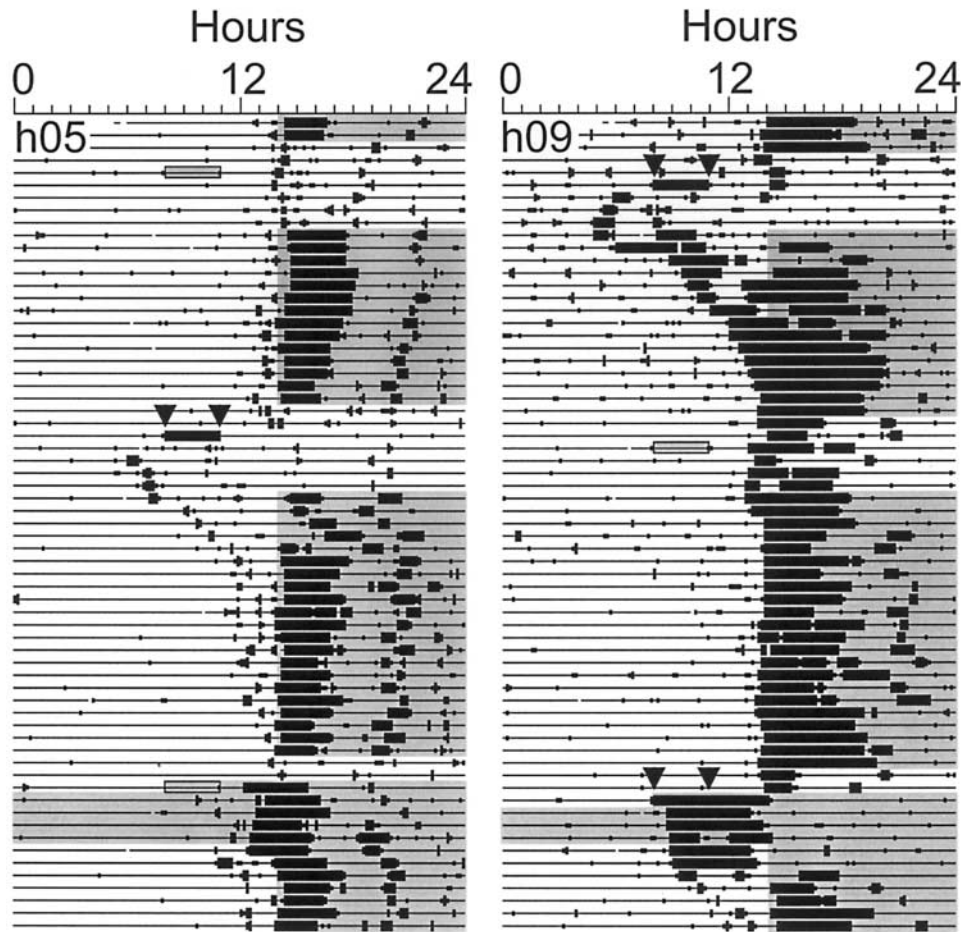


Figure 2. Actograms from two hamsters (H5, H9) illustrating phase shift responses to 3 h dark pulses or step changes in lighting from constant light to constant darkness, with wheel open (arrowheads) or wheel locked during the pulse. Each line represents a day, plotted in 10 min time bins from left to right. Vertical deflections indicate bins during which wheel revolutions were registered. Shading represents lights-off. Dark pulses are also indicated by a rectangle or arrowheads. These were the only two hamsters that ran spontaneously throughout the dark pulses in the wheel-open condition, and both expressed very large phase shifts. Neither hamster shifted appreciably following dark pulses during which the wheels were locked. H9 ran continuously for 6 h following the light to dark step change and exhibited a large phase advance shift, whereas H5 did not have wheel access for the first 3 h of the step change and did not exhibit a large shift.

subjected to dark pulses at CT 6 with wheels either locked or open (counterbalanced for order). Behavioral state was scored by observation at 1-min intervals. Regression lines were fit to activity onsets to measure phase shifts.

The results were strikingly similar to those from the 2-day LL procedures (tests 1 and 2). In the wheel-open condition, hamsters averaged 71 ± 66 min of wake time and 43 ± 78 min phase advance shifts. Four hamsters were awake for 166 min or more, ran on average 4825 ± 1245 revolutions, and shifted 127 ± 119 min. The remaining hamsters ran only 44 ± 55 revolutions, in 22 to 48 min of spontaneous wake time, and shifted only 13 ± 22 min. In the wheel-locked condition, spontaneous wake time averaged 38 ± 20 min (14 to 86 min) and

phase shifted only 16 ± 52 min. Time in LL, after 2 days and for up to 20 days, had no influence on the duration of the acute behavioral waking response to darkness or on the magnitude of associated phase shifts.

Test 6: Effect of Sleep Deprivation by Gentle Handling on Phase Shifts to Dark Pulses

The results of tests 1 to 5 indicate that most of our hamsters tended to sleep through most of the 3 h dark pulses and phase shift little, whereas those few that did stay awake invariably ran and exhibited large shifts. To determine whether continuous running is necessary for these large phase shifts, the hamsters were reentrained to LD for 21 days and then exposed

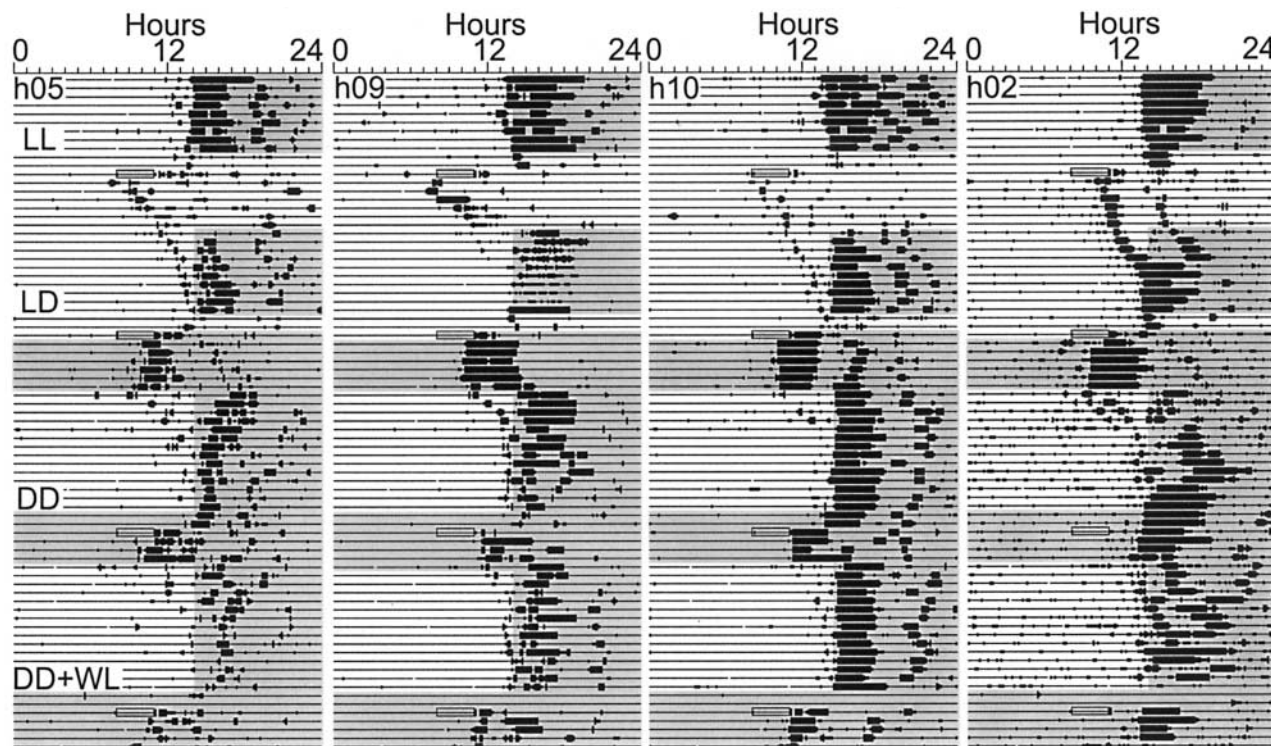


Figure 3. Actograms from four representative hamsters illustrating phase shift responses to 3 h of sleep deprivation (SD) on the third day of constant light (LL), following a step change in lighting from LL to constant darkness (DD) (i.e., LD), on the third day of DD and on the third day in DD with the wheels locked until after the deprivation procedure (DD+WL). H2 was one of only two animals that shifted less than 140 min to both SD procedures conducted in DD. See Figure 2 for plotting conventions.

to LL for 2 days. On day 3 of LL, beginning at projected CT 6, the hamsters received a 3 h dark pulse combined with sleep deprivation by gentle handling, with the running wheels locked. LL was then resumed for 4 days. Phase shifts measured on the second day after deprivation averaged 261 ± 110 min (Figs. 1, 3). The largest shift (7.3 h) was comparable to the largest shifts exhibited by hamsters that ran continuously during dark pulses in tests 1 and 2. These results indicate that continuous running is not necessary for maximal phase shifts to dark pulses.

Test 7: Role of Lights-On after Sleep Deprivation: Step Change in Lighting

Phase shifts induced by the sleep deprivation procedure in test 6, and expressed by the two hamsters that stayed awake spontaneously and ran throughout the dark pulse in tests 1 and 2, were unusually large for behavioral manipulations of 3 h duration. Previous reports from this and other laboratories indicate that the maximal shift to a 3 h wheel confinement in hamsters is rarely more than 240 min (in fact, none exceed-

ing that size had been observed previously in this laboratory) and that the mean shift is usually in the 100 to 200 min range (e.g., Bobrzynska and Mrosovsky, 1998; Mistlberger et al., 1996; Antle and Mistlberger, 2000). Most of these studies were conducted in DD or using the Aschoff type II procedure for measuring phase shifts (the behavioral manipulation is conducted during the first 3 h of DD, which is initiated at various times in the LD cycle, and the lights remain off for several days; see Mrosovsky, 1996). In test 3, we examined whether lights-on after a 3 h dark pulse might suppress phase shifts to a potentially weak behavioral zeitgeber (i.e., <60 min of spontaneous waking in 3 h). We obtained no evidence that lighting after the dark pulse mattered. An alternative possibility is that rather than inhibiting a weak zeitgeber, lights-on after a dark pulse might potentiate the shift response to a strong behavioral zeitgeber (e.g., 3 h of enforced waking during darkness). Evidence consistent with synergy between successive nonphotic and photic pulses has previously been reported (Mrosovsky, 1991).

To test this idea, the hamsters were reentrained to LD and then exposed to LL for 2 days. On day 3 of LL,

the hamsters were sleep deprived for 3 h at projected CT 6. Lights were turned off during the sleep deprivation and remained off for 4 subsequent days. This procedure was associated with substantial phase advance shifts averaging 234 ± 53 min (range: 150 to 340 min). This was not significantly different from phase advances to sleep deprivation during a 3 h dark pulse (matched $n = 13$). These results indicate that lights-on after a 3-h dark pulse initiated 6 h before the usual active period plays no role in setting the magnitude of the phase shift produced by this procedure.

Test 8: Phase Shifts to Sleep Deprivation in DD

In the Aschoff type II procedure used previously to examine phase-shifting effects of sleep deprivation in hamsters, deprivation was conducted in the dark, beginning at ZT6 (i.e., after 8 h of light) (Antle and Mistlberger, 2000). In tests 1 to 7, dark pulses, with or without sleep deprivation, were initiated after ~56 h of light. Test 7 demonstrated that lights-on after sleep deprivation in the dark does not contribute to the resulting phase shift. In test 8, we examined whether LL prior to sleep deprivation was necessary to induce very large phase shifts. The hamsters were reentrained to LD, then maintained in DD for 2 days. On day 3 of DD, the hamsters were sleep deprived for 3 h at projected CT 6 and then left undisturbed for 4 more days in DD. This procedure resulted in a group mean phase shift of 152 ± 91 min, which was substantially and significantly smaller than the mean phase shifts observed in tests 6 and 7 ($p < 0.05$, matched $n = 13$), when hamsters were exposed to LL prior to sleep deprivation. These results indicate that ~56 h of LL potentiates the phase shift response to sleep deprivation conducted with the lights off.

Test 9: Effect of Prior Wheel Access in DD on Phase Shifts to Sleep Deprivation

LL suppresses spontaneous wheel running in nocturnal rodents (e.g., Aschoff, 1960). The level of spontaneous wheel running in the subjective night is thought to exert a feedback effect that modulates the period of the circadian pacemaker (e.g., Yamada et al., 1986). Feedback may also regulate the responsivity of the pacemaker to nonphotic inputs, such as provided by sleep deprivation. Conceivably, reduced feedback in LL may increase responsivity of the clock. To test this, the hamsters were reentrained to LD and then

maintained in DD with their running wheels locked. On day 3 of DD, the hamsters were sleep deprived for 3 h as described previously and running wheels were subsequently unlocked. Activity onset on the day prior to sleep deprivation in test 8 (DD, wheel open) was used as the baseline reference for measuring phase shifts. This procedure is valid if τ is not substantially affected by 2 days without wheel access. In hamsters, τ is very close to 24 h during the first few days of LL and DD. In a previous study, we detected no effect of wheel availability on τ at any time point over several months in DD, using drinking activity as a behavioral measure (Mistlberger and Marchant, unpublished data, 1995). One study reports that τ in hamsters correlates negatively with the amount of daily wheel running, but the effect was characterized as weak and inconsistent (Mrosovsky, 1999) and would likely contribute very little to circadian phase in only 2 days of DD.

Twelve hamsters produced usable data in both tests 8 and 9. Phase shifts in this group averaged 132 ± 65 min in test 8 (wheel open in DD prior to deprivation) and 162 ± 64 min in test 9 (wheel locked in DD prior to deprivation) ($p < 0.05$). Phase shifts in both DD conditions were significantly smaller than in the LL conditions (tests 6 and 7) ($p < 0.05$).

DISCUSSION

The results of this study permit the following substantive conclusions: (1) 3 h dark pulses in the mid-subjective day typically stimulate only transient arousal from sleep and do not induce phase shifts unless waking is sustained for most of a pulse. In these latter cases, waking is invariably accompanied by continuous wheel running. These results reinforce the view that phase shifts to periods of darkness confined to the mid-subjective day in LL are mediated by "nonphotic" inputs to the clock, generated by acute behavioral responses to lights-off. (2) Continuous wheel running is not necessary for the induction of phase shifts in response to 3-h dark pulses in the mid-subjective day; enforced waking is sufficient. This result bolsters the evidence that the pacemaker can be shifted by sustained waking without intense exercise (Antle and Mistlberger, 2000). (3) Phase shifts to sleep deprivation in the dark are greatly potentiated by ~2 days preexposure to LL. (4) Suppression of wheel running by light may contribute to this synergistic effect of LL on shift magnitude. (5) By the third day of LL, more

than 3 h of dark is required to restore pacemaker sensitivity to light.

These conclusions may be particular to dark pulses and behavioral manipulations confined to the mid-subjective day. The results of another recent study indicate that phase advances to 6 h dark pulses initiated at CT 11 are not correlated with the level of wheel running during the pulse (Dwyer and Rosenwasser, 2000). Given that wheel running and wake time appear to be highly correlated in hamsters, it is likely that phase shifts would also be uncorrelated with wake time. This is consistent with other work indicating that the circadian clock is relatively (e.g., Mrosovsky et al., 1992), although probably not entirely (e.g., Mistlberger and Holmes, 2000), insensitive to nonphotic inputs early in the subjective night.

Methodological Issues

This study used a within-subjects design in which the same animals were tested on nine occasions over ~8 months of age. This raises the possibility that some aspects of the results may be related to prior lighting history, prior exposure to nonphotic stimuli, or aging. However, previous studies, as well as the present results, provide no evidence that these factors played a major role in determining the presence or magnitude of phase shifts. Recent studies that compared phase shifts to nonphotic stimuli (novelty-induced running or dark pulses) across multiple tests, over up to 8 months, in DD or LL alternating with LD, detected no significant change in shift magnitude (Weisgerber et al., 1997; Duncan and Deveraux, 2000). Our own data are consistent with stability of nonphotic resetting over this age range. Animals that were spontaneously awake and ran during the dark pulses or the LL to DD transitions in tests 1 to 3 showed very large phase shifts within the range of shifts induced by sleep deprivation in tests 6 and 7. In tests 8 and 9, mean phase shifts to sleep deprivation on day 3 of DD were comparable in size to phase shifts induced by sleep deprivation or novelty-induced running on day 1 of DD in previous studies (the Aschoff type II procedure) (Mistlberger et al., 1996; Antle and Mistlberger, 2000). These shifts were ~100 min smaller than shifts induced by sleep deprivation on day 3 of LL (tests 6 and 7). The close proximity of tests 6 to 9 argues further against an important role for time, as opposed to lighting, in the differences across these conditions.

The external time of activity onset (CT 12) on the day prior to sleep deprivation did not differ signifi-

cantly in LL (tests 6 and 7) and DD (test 8). This indicates that the free-running period was quite similar over the first 2 days of constant conditions, regardless of lighting, which ensures that the sleep deprivations in LL and DD were conducted at comparable circadian phases and that differences in phase shift magnitude between the two lighting conditions were not the result of experimental manipulations occurring at substantially different points on the nonphotic phase response curve.

Mechanisms

In a study of mice, lesions of the intergeniculate leaflet (IGL) blocked entrainment to a daily schedule of forced running, a procedure that ensures continued wakefulness (Marchant et al., 1997). These lesions eliminate a neuropeptide Y (NPY) containing pathway to the suprachiasmatic nucleus (SCN), the site of the primary circadian pacemaker. In hamsters, IGL lesions or antibody to NPY can block phase shifts to procedures that stimulate running or arousal (Biello et al., 1994; Janik and Mrosovsky, 1994; Wickland and Turek, 1994; Maywood et al., 1997). In these studies, behavioral state, independent of running, was typically not monitored; thus, it is conceivable that ablated animals fail to shift because of changes in their ability to maintain continuous waking. Nonetheless, it is reasonable to predict that procedures that interfere with NPY transmission will also block phase shifts to sleep deprivation by gentle handling.

A sleep deprivation perspective on nonphotic shifting suggests that sleep factors are additional candidate signals that convey the effects of enforced waking to the circadian clock. We have recently found that A1 receptor agonists for the putative sleep factor adenosine can induce phase shifts similar to sleep deprivation, and that caffeine, an adenosine A1 antagonist, stimulates waking without phase shifts and attenuates phase shifts to continuous running (Antle et al., 2001). Whether adenosine mediates phase shifts to sleep deprivation and how it might interact with known nonphotic inputs to the SCN pacemaker (e.g., NPY and 5HT; reviewed in Mistlberger et al., 2000) remains to be determined.

The mechanism by which LL potentiates phase shift responses to sleep deprivation also remains to be elucidated. Time in DD has been shown to influence the magnitude of phase shifts in response to light pulses in at least a few studies (Shimomura and Menaker, 1994; Mistlberger and Holmes, 2000), but the

effects of time in LL on nonphotic shifts has been little studied. We are aware of only one other study that has reported potentiation of "nonphotic" phase shifting (to 8-OH-DPAT or saline injections) by exposure to LL (J. D. Glass, personal communication, June 2000). One possibility is that as little as 2 days of LL significantly attenuates pacemaker amplitude, and that this results in enhanced phase displacements to standard nonphotic perturbations (for a discussion of analogous effects in humans, see Czeisler, 1995). Suppression of pacemaker amplitude in LL could follow in part from reduced behavioral feedback effects on the pacemaker due to inhibition of activity by LL and resulting attenuation of the amplitude of the overt rest-activity cycle. If a change in pacemaker amplitude is the explanation, then phase shifts to all types of zeitgebers should be similarly enhanced. Also, brighter light might be expected to result in larger shifts (e.g., Dwyer and Rosenwasser, 2000). Alternatively, attenuation of feedback effects of activity on the clock could sensitize the nonphotic input pathway, resulting in selective potentiation of nonphotically induced phase shifts.

An alternative conception is that the potentiation of phase shifts in LL is due to lights-off during the deprivation, not to an effect of prior light exposure on pacemaker amplitude or specific input paths to the clockworks. Given that dark pulses without sustained waking did not induce shifts, this seems unlikely. Nonetheless, it seems reasonable to speculate that darkness raises the "level of arousal" during a sleep deprivation test. Previously, we found that sleep deprivation is much harder to accomplish (i.e., requires more interventions) when the lights are on (Antle and Mistlberger, 2000) and that the use of more interventions was predictive of smaller shifts. This may be due to a direct effect of light on arousal mechanisms, an idea in the field of chronobiology that dates at least to Aschoff (1960). To extend this analysis, studies will need to operationalize the concept of level of arousal in more physiological terms (e.g., autonomic, endocrine, or cortical activation).

The lack of effect of subsequent lighting on the magnitude of phase shifts to spontaneous running or forced waking was a striking feature of the data. Phase shifts to 3 h of sleep deprivation in the Aschoff type II protocol are expressed within 4 h of the onset of deprivation, as demonstrated using photic induction of Fos in SCN neurons as a circadian phase marker (Antle and Mistlberger, 2000). Therefore, when LL is resumed after a 3-h dark pulse combined with sleep deprivation

or continuous running, the light is presumably striking the clock at a photosensitive phase and should therefore be expected to induce a shift in accordance with the photic phase response curve. Whether the shift is initially a delay or an advance would depend on how far the clock is shifted after 3 h. We do not presently know the temporal dynamics of wake-induced phase advances that exceed 3 h in magnitude, but the results suggest that the clock was insensitive to light after the sleep deprivation. Peripheral and/or central components of the photic input pathways, to the level of the clock cells, may be desensitized by prior LL, and a 3-h dark pulse may be insufficient to reverse this desensitization. Desensitization caused by prior exposure to brief pulses of light in hamsters free-running in DD appears to involve response saturation (a ceiling on the integrated response to two or more light pulses presented at interstimulus intervals of less than 1 h) (Nelson and Takahashi, 1999), but response adaptation (denoted by a shift in the dose-response curve to lights-on) may also be a factor when light is prolonged.

Recent data suggest that part of the phase advance induced by a 6 h dark pulse initiated at CT 8 is due to the onset of light (signaled by glutamate activation of N-methyl-D-aspartate receptors in the SCN) at what would have been CT 14, a time when light normally has a phase-delaying effect (Piggins et al., 2000). Presumably, in those hamsters the clock was rapidly phase advanced by the initial arousal response at dark onset and by the absence of light in the projected early subjective night (the phase delay portion of the photic phase response curve), causing LL to resume in the phase advance portion of the photic phase response curve. Together with the results of the present study, this suggests that recovery of responsiveness to lights-on following 2 or more days of LL requires between 3 and 6 h of darkness.

Unresolved Conceptual Issues

The main results of this study provide support for the idea that being awake in the usual sleep period can shift the clock. Logically, however, phase shifts could result from either the presence of wake or the absence of sleep. The latter alternative would require that sleep in the hamster's subjective day normally exert a continuous phase-delaying effect on the circadian clock, which, if prevented by spontaneous or enforced waking, causes a relative phase advance. This problem may be experimentally tractable, as procedures for

inducing additional sleep in the subjective day could be informative. In humans, naps in the morning with the lights off are associated with phase delays, but midday naps produced no shifts and evening naps produced advances (Buxton et al., 2000). Additional studies using totally blind subjects could tease out any contributions of altered photic input during naps.

Perspective

The potency of enforced wakefulness as a zeitgeber in the absence of continuous wheel running provides a means of studying behaviorally induced clock resetting in cases where running is minimal or cannot be measured due to experimental treatments, age, species, or other variables. For example, in at least some studies, old hamsters run less and phase shift less in response to dark pulses (Van Reeth et al., 2001; but see Duncan and Deveraux, 2000). Melatonin treatment partially restores the phase shifting but not the running response (Van Reeth et al., 2001). However, it is conceivable that young and old hamsters may phase shift equivalently if they are prevented from sleeping during dark pulses. If so, melatonin may reverse an apparent deficit by altering the arousal response to darkness rather than the sensitivity of the clock to a nonphotic input. These considerations suggest that there may be no substitute for observing behavioral state to adequately interpret the effects of experimental treatments or constitutional variables on nonphotic clock resetting.

ACKNOWLEDGMENTS

This study was supported by an operating grant to the first author and a graduate fellowship to the third author from the National Science and Engineering Research Council of Canada.

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