Behavioral feedback regulation of circadian rhythm phase angle in light-dark entrained mice

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Mistlberger, R. E., and M. M. Holmes. Behavioral feedback regulation of circadian rhythm phase angle in light-dark entrained mice. Am J Physiol Regulatory Integrative Comp Physiol 279: R813–R821, 2000.—Induced and spontaneous wheel running can alter the phase and period (τ) of circadian rhythms in rodents. The relationship between spontaneous running and the phase angle (ψ) of entrainment to 24-h light-dark (LD) cycles was evaluated in C57BL/6j mice. With a wheel freely available, ψ was significantly correlated with the absolute (r = 0.32) and relative (r = 0.44) amount of activity during the first 2 h of the activity period. When wheels were locked during the first half of the night in LD and then unlocked in constant dark (DD), mice exhibited a delayed ψ and lengthened τ compared with mice that had wheels locked during the second half of the night. In DD, ψ correlated negatively with total daily activity. To evaluate if wheel running modulates the phase-resetting actions of LD, phase shifts to light pulses were measured at two time points in DD, when daily activity levels differed by 40%. Phase delays to light were 56% greater when activity levels were lower. However, in a counterbalanced follow-up experiment, phase advances and delays to light pulses were not affected by the availability of wheels, although an effect of time in DD was replicated. Spontaneous activity can regulate ψ and τ without altering the response of the pacemaker to light.

wheel running; entrainment; nonphotic zeitgeber; phase shifts; light pulses

For most mammalian species, the daily light-dark (LD) cycle is the dominant entrainment cue (zeitgeber) for synchronizing circadian rhythms of behavior and physiology to local time. However, the circadian timekeeping system in mammals also exploits nonphotic cues to coordinate the organism with its environment. Depending on the species, these zeitgebers may include daily cycles of food availability, ambient temperature, or social interactions (1, 8, 11, 18, 37). A more recently characterized, and unexpected, nonphotic zeitgeber is the organism’s own behavior. In constant light or dark (DD), free-running circadian rhythms in several rodent species can be phase shifted or entrained by the induction of running or arousal during the usual rest phase of the rest-activity cycle (e.g., Refs. 6, 10, 15, 16, 23, 24, 38). In addition, the period (τ) of free-running rhythms can be modulated by spontaneous running in a wheel; τ is shortened by access to a running wheel and is negatively correlated with the daily amount of wheel running or with the relative amount of activity occurring early in the active period (α) (7, 21, 41, 42). This adjustment of the rate of clock cycling by neural or endocrine correlates of spontaneous locomotor activity defines a feedback pathway from a behavioral “hand” of the clock to its molecular gears.

Nonphotic inputs to the circadian clock have adaptive significance only if they contribute in some way to setting (or resetting) the phase angle (ψ) of entrained circadian rhythms. Because most animals are exposed to daily photic cues in their natural habitats, the importance of nonphotic zeitgebers therefore turns on whether or not they are sufficiently potent to modulate entrainment to LD cycles. Several studies have shown that ψ can be significantly modified by daily schedules or single episodes of behavioral activation induced by confinement to a novel wheel, social interactions, or feeding (4, 11, 12, 17, 35). However, there are no reports yet of whether variations in spontaneous, clock-controlled locomotor activity might also contribute to phase control. Given that a short free-running τ in mice is associated with a concentration of activity early in α (7) and with an advanced phase of photic entrainment (28), the following predictions can be made: 1) during stable entrainment to LD, ψ may be related to the distribution of spontaneous activity within α and 2) ψ may be altered by manipulations of the distribution of spontaneous activity within α. The results of the present study are consistent with these predictions.

There are several possible mechanisms by which nonphotic cues might alter ψ. One possibility is that ψ is the net result of multiple daily phase shifts induced by photic and nonphotic inputs in succession. Stable ψ might thus be predictable from the known photic and nonphotic phase-response curves (PRCs; a plot of the relationship between the circadian phase at which a stimulus is applied and the direction and magnitude of the resulting phase shift).

Alternatively, nonphotic cues may adjust ψ by modulating pacemaker τ or the response of the pacemaker to photic inputs. In hamsters and mice, phase shifts to light pulses can be attenuated by concurrent behav-

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ioral activation at circadian phases when behavioral activation alone has little or no phase-shifting effect (19, 22, 29). These results raise the possibility that spontaneous wheel running activity just after lights-out or just before lights-on may regulate the gain of photic phase resetting in mice. The results of the present study, however, do not provide support for this hypothesis.

METHODS

Subjects and Apparatus

Male C57BL/6j mice were obtained at 8 wk age (Charles River, Quebec, Canada) and housed in separate plastic cages (45 × 25 × 20 cm) equipped with running wheels (17 cm diameter), contact drinkometers, and wire mesh floors over litter trays in a climate-controlled vivarium. Wheel running and drinking activity were monitored continuously by microcomputer and stored on disk at 10-min intervals. Illumination during lights-on (~100 lx) was provided by overhead fluorescent fixtures.

Procedures

Experiment 1. Correlational study of ψ and activity. A group of 39 mice was maintained in a 12:12-h LD cycle for 14 days and then in DD for 1 day. The onset of nocturnal α (i.e., ψ) was quantified and correlated with the level and distribution of wheel running revolutions during the last 5 days of LD and the first day of DD.

Experiment 2. Experimental study of ψ and activity. A second group of 40 mice was maintained in DD for 32 days, in LD for 40 days, then in DD again for 21 days. An infrared viewer was used for checking food and water in the dark as necessary. Free-running τ was measured before and after LD. During the last 20 days of LD, the home cage running wheel was locked from zeitgeber time (ZT) 11 to 18 (where ZT12 is dark onset, by convention) in 20 mice and from ZT18 to 11 in the other 20 mice. Wheels were locked by inserting a metal rod through the bars of the cage top and the running wheel. The rod could be installed or removed in a few seconds without disturbing the mice. Activity restriction ended on the last day of LD. The time of α onset (i.e., ψ) was quantified for the 5 days before the activity restriction procedure, the first 5 and last 5 days of the restriction schedule, and the first day of DD.

Experiment 3. Correlational study of photic resetting and activity. A group of 20 mice was maintained in DD for 170 days. The mice were subjected to light pulses (40 lx, 20 min) at circadian time (CT) 16 (where CT12 is the onset of α) on DD days 54 and 156. Light pulses were applied by placing the subjects’ home cage in a shielded light box in the recording room. Mean activity levels and τ during the week before each light pulse were calculated. Phase shifts in response to the two light pulses were also measured and compared.

Experiment 4. Experimental study of photic resetting and activity. A group of 20 mice were maintained in DD for 151 days. Wheel running and drinking were monitored. During the first 65 days, the running wheels were locked in one group of 10 mice and left open in the other group. On day 47, all of the mice received a light pulse (4 lx, 10 min) at CT16, using a light-box as in experiment 3. On day 65, the wheel-lock condition was reversed. On day 82, a second light pulse was delivered at CT16. On day 99, a light pulse was delivered at CT23. On day 116, the wheel-lock conditions were reversed again, and on day 133, a final light pulse was delivered at CT23. For each wheel-access condition, the timing of light exposure was determined by regression lines fit to drinking data (see below).

Data Analysis

Activity data were transferred to a Macintosh computer for plotting and analyses using Circadia (Behavioral Cybernetics, available from the author for correspondence), SPSS (SPSS Inc.), and CricketGraph (Computer Associates International). For experiments 1–3, the onset of α each day was defined as the first 10-min bin in which wheel revolutions exceeded 25 or 50 after an interval of 240 min during which this threshold was not exceeded. Occasional spurious onsets were deleted (i.e., onsets deviating by >3 h from the previous day’s onset). ψ was expressed as the difference in minutes between the average time of α onset and the time of lights-off (where positive values represent α onset in advance of lights-off). The criteria for detecting α onsets using drinking data (experiment 4) were modified such that the threshold levels were set individually for each animal. In most cases there were still many “spurious” onsets. Consequently, computer-identified onsets retained for line fits were based on careful inspection of the data by two raters. A typical example of the results of this “subjective” method is illustrated in Fig. 6B and demonstrates a good correspondence with onsets fit by computer algorithm to wheel running data where available. To estimate τ in DD, a least-squares regression line was fit to α onsets. Phase shifts to light pulses in DD were measured by calculating the displacement between regression lines fit to α onsets for 7–10 days before and 7–10 days after a light pulse, excluding the first 3–5 days after the light pulse to ensure that the phase-shifting process was complete. Associations between wheel running, τ, and ψ were evaluated using the Pearson bivariate correlation procedure. In the text, means are reported ± SE.

RESULTS

Experiment 1. The Relation Between Activity and ψ

In LD, nocturnal wheel running began on average 18 ± 2 min after dark onset. Mean activity levels across animals ranged from 1,100 to 12,860 wheel revolutions/day (group mean = 4,931 ± 474 revolutions/day) and did not correlate significantly with ψ. Inspection of the scatterplot suggests that the correlation coefficient may have been substantially influenced by 3 of 40 data points in which very high levels of wheel running were associated with a negative ψ (Fig. 1A).

To determine if ψ was related to the amount of activity early in α, activity was quantified during the first 2 h after α onset (CT12–13). This analysis revealed that a more positive ψ was associated with higher levels of activity early in α (r = 0.32, P = 0.04).

To determine if ψ was related to the relative distribution of activity within α, the percentage of total daily activity occurring from CT12 to 13 was calculated. Again a significant positive correlation was revealed; ψ was increasingly positive as more of the total daily wheel running was concentrated within the first 2 h of α (r = 0.44, P = 0.004; Fig. 1B).

To determine if the LD cycle masked a stronger relation between ψ and activity, the time of α onset was correlated with the percentage of total activity occur-
ring from CT12 to 13 on the first day of DD. The correlation was positive and significant, but not appreciably more robust, than for the LD data \((r = 0.46, P = 0.003); \text{Fig. 1C}\).

Experiment 2. Effects of Activity Restriction on \(\psi\) and \(\tau\)

Experiment 1 revealed that \(\psi\) tends to be more positive when spontaneous activity is concentrated early in \(\alpha\). If there is a causal relationship between \(\psi\) and the distribution of activity within \(\alpha\), then restricting spontaneous activity to the first or the second half of the night should differentially affect \(\psi\). Running wheels were locked from ZT11 to 18 or from ZT18 to 11 (Fig. 2). Visual observation of the mice and inspection of the activity records revealed that the locking/unlocking procedure at ZT11 produced little or no behavioral activation. The two groups showed a similar average \(\psi\) before wheel restriction \((-16 \pm 2 \text{ and } -15 \pm 1 \text{ min}, \text{respectively})\). During wheel restriction, mice with wheel access during the first half of the dark period showed a small phase advance of activity onset, which was significant for the last block of 5 days of wheel restriction, by comparison with onsets before wheel restriction \((\text{mean advance} = 6 \pm 2 \text{ min}; t = 3.81, P < 0.001)\). Mice with wheel access during the second half of the night began to run as soon as the wheel was unlocked at ZT18.

On the first day of DD, with wheels freely available, \(\alpha\) onset began 31 \pm 7 min \((\text{paired } t = 6.81, P < 0.001 \text{ vs. baseline})\) before the usual time of lights-off in mice that had been permitted to run in the first half of the night and 23 \pm 9 min \((\text{paired } t = 2.4, P < 0.05 \text{ vs. baseline})\) after the usual time of lights-off in mice that had been permitted to run in the second half of the night \((\text{Fig. 3A})\). These onset times differed significantly between groups \((t = 4.66, P < 0.001)\) and also by
comparison with mean α onset time on the first day of DD in the 39 mice used in experiment 1 (14 ± 7 min; \( t = 3.1, P < 0.01 \) and \( t = 3.5, P < 0.01 \), compared with early and late wheel-access groups, respectively; Fig. 3B).

During the first week of DD, mice that had wheel access in the first half of the night in LD exhibited significantly longer \( \tau \) values than mice that had wheel access during the second half of the night (23.81 ± 0.04 vs. 23.65 ± 0.03 h, \( t = 3.41, P = 0.002 \), Fig. 3B). By days 10–18 in DD, the difference was smaller and not statistically significant (23.79 ± 0.06 vs. 23.69 ± 0.03 h, \( P > 0.1 \)).

Correlation coefficients were calculated between mean daily activity and \( \tau \) during DD before and after the LD wheel-restriction condition. A significant (\( P < 0.01 \)) negative correlation was evident at all three time blocks assessed, including week 3 before LD (\( r = -0.40 \)) and days 1–8 (\( r = -0.51 \)) and 10–18 (\( r = -0.59 \)) after LD (Fig. 4).

**Experiment 3. Relationships Between Activity, \( \tau \), and Phase Shifts to Light**

Twenty mice received two light pulses separated by 102 days in DD. Group mean activity levels declined from 12,230 ± 1,542 wheel revolutions/day during the week before the first light pulse (DD days 47–54) to 7,415 ± 942 revolutions/day during the week before the second light pulse (DD days 149–156; \( t = 4.17, P = 0.006 \); Fig. 5A). Phase delay shifts to light pulses at CT16 were significantly smaller at the first time point (−2.43 ± 0.27 vs. −3.81 ± 0.33 h, respectively; \( t = 3.61, P = 0.002 \); Fig. 5B). The magnitude of the shifts did not correlate significantly with the mean level of activity during the prior week at either time point (\( r = 0.07 \) and 0.18, respectively, \( P > 0.1 \)). Despite the significant decline in mean daily activity over time, there was no significant difference in mean \( \tau \) at the two time points (23.79 ± 0.04 and 23.86 ± 0.07 h, respectively; \( t = 1.04, P > 0.1 \); Fig. 5C). However, mean daily activity levels were significantly correlated with \( \tau \) before the first light pulse (\( r = -0.48, P < .01 \)), although this was not the case for the week before the second light pulse (\( r = -0.07 \)).

**Experiment 4. Phase Shifts to Light With and Without Wheel Access**

To determine if the increase in the mean size of phase delay shifts in experiment 3 might be caused by decreased activity levels, phase shifts to light pulses at CT16 and CT23 were assessed twice each, once after the wheel was locked for 3 wk and once when the wheel was freely available. Light pulses at CT16 induced group mean phase delay shifts of −2.30 ± 0.14 h in the wheel-unlocked condition, and −2.15 ± 0.15 h in the wheel-locked condition (paired \( t = 1.27, P > 0.1 \); Figs. 3A and 3B).
Light pulses at CT23 induced group mean phase advances of 0.88 ± 0.16 h in the wheel-unlocked condition, and 0.99 ± 0.17 h in the wheel-locked condition (paired t = 0.48, P > 0.1; Figs. 6 and 7). In the wheel-unlocked condition, phase shifts measured using wheel running data did not differ significantly from shifts measured using drinking data (paired t = 0.60, P > 0.1).

When data from the wheel-locked and wheel-opened conditions were combined at each of the two time points (days 42 and 84 in DD), mean phase delay shifts were significantly larger at the second time point (−2.03 ± 0.13 vs. −2.42 ± 0.14 min, respectively; 1-tailed paired t = 2.01, P = 0.03), which is consistent with predictions based on the results of experiment 3. However, there was no difference in mean phase advance shifts to light pulses at CT23 on days 102 and 138 of DD (1.1 ± 0.16 vs. 0.8 ± 0.17, t = 0.5, P > 0.1).

Wheel running levels (5-day means before the light pulse days in those mice with wheel access) also did not differ across time when data were compared from the first two light pulses (days 42 vs. 84; 7,616 ± 2,368 vs. 8,035 ± 1,748 revolutions/day, respectively), the second two light pulses (days 102 vs. 138; 6,973 ± 1,594 vs. 7,877 ± 1,743 revolutions/day, respectively), or the first two and second two combined (days 42 + 84 vs. days 102 + 138; 7,815 ± 1,458 vs. 7,403 ± 1,152 revolutions/day). This lack of effect of time in DD on wheel running levels may be due to the intermittent wheel access.

**DISCUSSION**

Previous studies have shown that 1) free access to a running wheel in DD shortens τ in rats (41, 42) and mice (7, 21), 2) the total amount of daily running correlates negatively with τ in rats (34) and hamsters (26), and 3) the relative amount of wheel running early in α correlates negatively with τ in mice (7). The present study extends these observations in two ways. First, we found that variations in the amount of spontaneous daily wheel running can be significantly associated with variations of τ in DD in mice. In one sample of 40 mice (experiment 2), the greater the average number of wheel revolutions per day, the shorter the τ. In another sample of 20 mice (experiment 3), the same significant association between the level of activity and τ was evident at one time point, although not at a second time point 102 days later. This may be due to the reduced range of activity levels at the second time, a factor suggested to account for inconsistent results found across separate groups of hamsters (26). Variable results may also be expected if the effect size is small and if τ can be changed by aftereffects or occasional variations in the distribution of activity within α caused by cage servicing or other sources of behavioral arousal (e.g., Ref. 39).

Second, we found a significant relationship between ψ and the distribution of spontaneous wheel running within α. The greater the absolute or relative amount of activity early in α, the more advanced the ψ. On the basis of the correlation coefficients obtained, 10–21% of the variance of ψ can be attributed to variations in the distribution of activity, a significant, albeit modest, proportion. This relationship was of similar magnitude when the first day of DD was used to assess phase, indicating that the LD cycle did not significantly mask pacemaker phase in the entrained state. This led us to predict that ψ would be relatively advanced if spontaneous wheel running was restricted to the first half of the night and relatively delayed if running was restricted to the second half of the night. The results of experiment 2 were consistent with this prediction.
The relationship between the distribution of wheel running within α and the timing of α onset relative to the LD cycle could reflect a direct effect of activity on state parameters of the clock (e.g., τ) or an indirect effect mediated by modulation of the photic input pathway to the clock. Evidence for the latter, indirect mechanism follows from observations that wheel running stimulated by confinement to a novel wheel or by drug injection can attenuate phase shifts to light pulses in hamsters and mice in DD (19, 22, 29). According to nonparametric entrainment theory, light exposure at the beginning and the end of the photoperiod (i.e., dawn and dusk) is sufficient to mediate entrainment and is likely of predominant importance (28). Indeed, some nocturnal animals require only a very brief exposure to evening light every few days to remain synchronized to local time (5). If behavioral activation can inhibit the response of the pacemaker to light pulses in DD, then it is conceivable that spontaneous, clock-controlled activity may modulate the response of the pacemaker to the light that serves as “dawn” or “dusk” in LD. We evaluated this hypothesis in two ways. First,
in experiment 3, we measured the magnitude of phase shifts to a light pulse delivered at two times in DD, separated by just over 3 mo. During the week before the second light pulse test, mean daily activity levels were reduced by 40% compared with the first test, whereas the mean phase delay shift to light was enhanced by 56%. These results are consistent with the idea that the gain of photic phase resetting can be modulated by the level of spontaneous wheel running activity.

However, in experiment 4, using a within-subjects counterbalanced design, we found no effect of wheel access on the size of phase delay or phase advance shifts to light, although there was an effect of time in DD (i.e., days 42 vs. 84) on the size of delay shifts, as in experiment 3. This suggests that the increased size of light-induced phase delays after the second light pulse test in experiment 3 (and similar reports of increased phase shifts to light with time in DD in hamsters; Refs. 32 and 33) was probably not a direct consequence of the reduced level of spontaneous wheel running at that time. Instead, it could reflect changes in the photic PRC caused by gradual damping of pacemaker amplitude or altered internal coupling between component oscillators of the pacemaker (a possible basis for amplitude changes), which theoretically should increase the magnitude of phase resetting responses. Changes in pacemaker amplitude or internal coupling could be caused by one or more of the following: the extended absence of an entraining zeitgeber, the (modestly) increased age of the animals, or the reduced level of wheel running, as wheel running has been shown to enhance sleep-wake rhythm amplitude (40). Photoreceptor adaptation with time in DD also cannot be ruled out but seems an unlikely explanation given that the first light pulse in experiment 3 did not occur until day 54 of DD.

The failure of wheel access to affect the size of phase shifts to light suggests that spontaneous wheel running alters $\psi$ by virtue of direct phase or $\tau$ modulation of the pacemaker, independent of the light input pathway. Evidence for $\tau$ modulation was obtained in experiment 2; mice allowed to run in wheels only during the first half of the night exhibited significantly shorter $\tau$ values during subsequent DD than mice allowed to run only during the second half of the night. Nonparametric entrainment models predict that a shorter $\tau$ should result in an advance and a longer $\tau$ a delay of $\psi$, thus the group difference evident in $\psi$ could be explained by activity-induced changes in $\tau$ (28).

Changes of $\tau$ after a single exposure to a photic or nonphotic zeitgeber and aftereffects on entrainment to these zeitgebers, alone or in combination, have been noted in previous studies (e.g., Refs. 13, 17, 25, 27, 39). Generally, light pulses that advance the clock shorten $\tau$, whereas pulses that delay the clock lengthen $\tau$ (27). Quantitative simulations suggest that this relation may serve to increase the stability of $\psi$ (3). Phase shifts and $\tau$ changes induced by nonphotic stimuli, however, do not show this relationship, as both phase advance and phase delay shifts are associated with $\tau$ lengthening in some studies (e.g., Ref. 25). A lengthening of $\tau$ after an advance is not conducive to stable entrainment, thus $\tau$ changes caused by nonphotic stimuli likely serve a different function (3). This is not surprising. Given that spontaneous activity is presumed to be a hand of the clock and a clock cannot entrain (by definition) to itself, a role for activity-induced $\tau$ changes in phase stabilization would not, a priori, be expected.

The sensitivity of pacemaker $\tau$ (and therefore phase) to concentrations of activity at certain circadian phases more likely reflects the adaptive value of adjusting phase to coordinate behavior optimally with significant events, such as encounters with mates or food sources, that may occur at particular times of day (e.g., Ref. 11). Small phase adjustments caused by variations in levels of activity early or late in the night are evidently not at odds with a general requirement for mice to remain predominantly nocturnal. Very large (3–12 h) phase shifts that can be induced by activity stimulated in the middle of the sleep period in hamsters demonstrate the potential magnitude of nonphotic effects (e.g., Refs. 9 and 35), but the presence of photic cues in natural habitats likely prevents such dramatic phase inversions (35).

In two previous studies, running stimulated by confinement to a novel wheel for 30–50 min was shown to attenuate phase advance shifts to 15-min light pulses of 8–40 lx (19, 29). The failure of wheel access to affect phase advance or delay shifts to 10-min, 4-lx light pulses in experiment 4 would appear to conflict with those earlier results. However, light pulses inhibit spontaneous wheel running in hamsters (19, 30). In previous studies, the use of a novel running wheel to stimulate activity was shown to override this “masking” effect of light (19). Inspection of the activity records from experiment 4 indicates that running during and for as much as 1 h after the light pulses was considerably lower than in the studies employing novel wheels. Pacemaker responses to light may be vulnerable to behavioral inhibition only for a limited temporal window during and immediately after the photic stim-
behavioral modulation of circadian phase angle

Perspectives

This study provides additional empirical evidence that variations in the expression of a clock-controlled behavior (e.g., spontaneous nocturnal wheel running) can alter functional properties of the clock (e.g., τ) in ways that would likely have adaptive significance in natural habitats (e.g., modulation of ψ). The results have broader implications that also merit attention. First, it is widely recognized that physical and biological phenomena can be altered by the process of observation and measurement. An example from the field of chronobiology is the dependence of circadian behavioral phenotype (i.e., nocturnality vs. diurnality and τ length) on the tool used to measure rhythmicity (e.g., Refs. 2, 7, 14, 21, 41, 42). Earlier demonstrations that novelty-evoked wheel running can alter pacemaker responses to light pulses raised the possibility that quantitative features of photic PRCs for rodents reflect the use of running wheels to measure pacemaker phase. Our finding that spontaneous activity in home cage wheels does not alter the magnitude of phase shifts to light attains this concern. Second, it is widely recognized that the timing and amplitude of circadian rhythms is altered in endogenous depression and other psychological disorders (31, 36). These disorders may also be characterized by changes in the amount and distribution of physical activity. Our finding that the amount and distribution of spontaneous wheel running can affect pacemaker phase in LD raises the possibility that changes in activity with mood may precipitate or exacerbate changes in circadian phasing. The role of such phase changes in the depressogenic process is unknown.

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