Daily Novel Wheel Running Reorganizes and Splits Hamster Circadian Activity Rhythms

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Abstract The phenomenon of splitting of locomotor activity rhythms in constant light has implied that the mammalian circadian pacemaker is composed of multiple interacting circadian oscillators. Exposure of male Syrian hamsters to novel running wheels also induces splitting in some reports, although novel wheel running (NWR) is better known for its effects on altering circadian phase and the length of the free-running period. In three experiments, the authors confirm and extend earlier reports of split rhythms induced by NWR. Male Syrian hamsters, entrained to LD 14:10, were transferred for 6 to 11 consecutive days to darkened novel Wahmann wheels at ZT 4 and were returned to their home cages at ZT 9. All hamsters ran robustly in the novel wheels. NWR caused a marked reorganization of home cage wheel-running behavior: Activity onsets delayed progressively with each additional day of NWR. After 11 days, activity onset in the nighttime scotophase was delayed by 7 h and disappeared completely in 2 hamsters (Experiment 1). After 6 to 7 days of NWR (Experiment 2), activity onset delayed by 5 h. Transfer of hamsters to constant darkness (DD) after 7 days of NWR revealed clearly split activity rhythms: The delayed nighttime activity bout was clearly identifiable and characterized by a short duration. A second bout associated with the former time of NWR was equally distinct and exhibited a similarly short duration. These components rejoined after 3 to 5 days in DD accomplished via delays and advances of the nighttime and afternoon components, respectively. The final experiment established that rejoining of activity components could be prevented by perpetuating the light-dark:light-dark cycle used to induce split rhythms. The data suggest that NWR causes selective phase shifting of some circadian oscillators and that component oscillators interact strongly in constant darkness.

Key words splitting, oscillator interaction, coupling, nonphotic

A multioscillator basis for mammalian circadian rhythms has been adduced through studies of photoperiodic control of activity duration (α), internal desynchronization, splitting, and most recently, in vitro electrical recordings of single SCN cells (Aschoff, 1965; Elliott and Tamarkin, 1994; Gorman et al., 1997; Illnerova, 1991; Liu et al., 1997; Pittendrigh and Daan, 1976). Each set of studies reinforces the idea that coherent circadian rhythms are generated from the interaction of coupled constituent oscillators with a range of free-running periods, τ. Although significant advances have been made in clarifying the neuroana-

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JOURNAL OF BIOLOGICAL RHYTHMS, Vol. 16 No. 6, December 2001 541-551

tomical and physiological substrates for rhythm generation and entrainment, the formal properties of oscillator interaction have received less sustained attention.

A major exception to this generalization is the study of split locomotor activity rhythms first reported in the arctic ground squirrel, *Spermophilus undulatus* (Pittendrigh, 1960) and elaborated further in studies of Syrian hamsters, *Mesocricetus auratus* (Pittendrigh and Daan, 1976). After prolonged (e.g., 60 days) exposure to constant light (LL), locomotor activity rhythms of some individuals dissociate into two components that free-run initially with different frequencies. When the two split activity components adopt an antiphase relationship (180 degrees apart), they free-run with a common frequency greater than that measured just prior to splitting. A comparable phenomenon is obtained in a day-active species exposed to low levels of light intensity (Hoffmann, 1971).

Exposure to constant lighting conditions is not the only manipulation capable of splitting mammalian circadian rhythms. Although not discussed in the text, Bruce's (1960) study of frequency demultiplication includes a single actogram of a hamster maintained in short cycles of 2 h light, 4.5 h dark (LD 2:4.5). In this record, two activity components 180 degrees apart were apparent for approximately 7 days before one of these components disappeared. Mrosovsky and Janik (1993) reorganized the activity rhythms of hamsters maintained in LD 14:10 by exposing them each afternoon to 3-h pulses of novel wheel running (NWR) in the dark (beginning 7 h before normal lights-off). When NWR was discontinued and hamsters were left in their home cages in constant darkness (DD), locomotor activity rhythms were split into two components that rejoined after 3 to 5 days, although this pattern was not equally clear in all records shown (e.g., #3802 in their Fig. 2). Nighttime activity onsets in LD 14:10 were also phase-delayed by several hours during NWR. Sinclair and Mistlberger (1997), using a different strain of hamster and a slightly modified protocol, found less compelling evidence of splitting after 17 days of NWR, although nighttime activity onset was delayed in some animals. Using the hamster strain employed by Mrosovsky and Janik (1993) and a modification of their experimental protocol, we here describe marked reorganizations of locomotor activity rhythms induced by three regimens of daily NWR.

MATERIALS AND METHODS

Animals and Husbandry

For all experiments, a subset of the same 24 male Syrian hamsters that were used in a separately reported study published in this issue (HsdHan: AURA; Harlan, Indianapolis, IN, USA) (Gorman et al., 2001 [this issue]), 5 to 6 weeks of age at acquisition, were housed with Sani-Chip bedding in polypropylene cages (48 × 27 × 20 cm) equipped with Nalgene (d = 34 cm) running wheels (Fisher Scientific, Pittsburgh, PA, USA). Food (Purina Rodent Chow #5001, St. Louis, MO, USA) and water were available ad libitum. Syrian hamsters were entrained to LD 14:10 (lights on 0500-1900 h; approximately 100 lux) for 3 weeks before an initial regimen of daily NWR was initiated.

Novel Wheel Running

Following entrainment to LD 14:10 (lights off = ZT 12), hamsters were transferred within the same room to Wahmann wheels (d = 34 cm) 0 to 15 min before lights were extinguished at 1100 h (ZT 4) EST. At 1600 h (ZT 9), the lights were turned on and hamsters were returned to home cages in the light over the next 15 min. Thus, during NWR, animals were exposed to an LDLD 6:3:5:10 light schedule. On one day, the darkened hamster room was entered through a light lock at hourly intervals from 1200 to 1600 h to record the number of novel wheel revolutions with the aid of a dim red light.

Analysis

Wheel-running activity in the home cage was monitored by Dataquest III software (Mini-mitter, Sun River, OR, USA) and compiled into 10-min bins. While in the novel wheels, activity patterns were not monitored, but the total number of wheel revolutions after the 5-h interval was recorded manually. Data analyses were carried out with Excel (Microsoft, Bellevue, WA, USA) and ClockLab software (Actimetrics, Evanston, IL, USA).

Activity onset was defined as the first time point in a scotophase in which a hamster ran more than 20 revolutions in a 10-min interval followed immediately by an additional 10-min interval with more than 20 wheel revolutions. Activity offset was defined as the last time point in a scotophase that the animal ran more than 20 revolutions and that was preceded immediately by a similar 10-min interval of activity. Activity duration (α) was calculated as the interval between activity onset and activity offset. An interval of inactivity was calculated as the difference between activity offset and the subsequent activity onset. The circadian period of activity onsets either in constant conditions (τ) or while exposed to a light-dark cycle (τ^*) was estimated with linear regression by determining slope of activity onsets over 4- to 7-day intervals. The phase angle of entrainment was determined from the average value predicted by the regression line and was expressed in relation either to the time of lights-off ($\psi_{lights\text{-}off}$) or lights-on ($\psi_{lights\text{-}on}$). When activity components were split, circadian parameters were calculated separately for activity bouts corresponding to the original 10-h dark period (i.e., the nighttime, *n*, activity bout) and to the 5-h interval of NWR (i.e., the afternoon, *a*, activity bout). The phase angle between components was defined as the difference between their respective activity onsets ($\psi_{n\text{–}a}$).

Experiment 1

Hamsters, 8 to 9 weeks of age, previously entrained to LD 14:10, were exposed to NWR in LDLD 6:3:5:10 ($n = 20$). After 11 days of these treatments, hamsters remained undisturbed in their home cages for 2 additional days under the same light conditions.

Experiment 2

Because 11 days of NWR in Experiment 1 phase-delayed nighttime activity onset more than expected on the basis of published studies, we next assessed whether more evenly split activity would be obtained after fewer days of NWR. Hamsters from Experiment 1, 12 to 13 weeks of age, were re-entrained to LD 14:10 for 14 days and exposed to NWR under LDLD 6:3:5:10 for 7 days ($n = 19$). Four additional hamsters, with identical light histories but no previous running-wheel exposure, were equipped with Nalgene wheels. In this experiment, these control hamsters were exposed to LDLD 6:5:3:10 without NWR. All hamsters were minimally disturbed during days of NWR except for a single retro-orbital bleeding conducted on the final day as part of another study. After the final day of NWR, 9 of the 19 hamsters, randomly selected, remained in this study and were exposed to constant darkness (DD) initiated during the subsequent 10-h scotophase (i.e., the lights remained off at 0500 h). Data from the remaining 10 hamsters are reported here only through the final day of NWR, after which they received a different light treatment described in a separate study (Gorman et al., 2001). Periods of the free-running rhythms of activity onset were calculated for each of the 9 hamsters during days 1 to 4 and 8 to 11 of DD.

Experiment 3

Because a distinctly and evenly split home cage running rhythm was obtained in Experiment 2, we asked whether these hamsters could be entrained to the LDLD cycle in effect during NWR. The same hamsters ($n = 20$ including 1 former control hamster from Experiment 2), 30 to 31 weeks of age, were re-entrained to LD 14:10 and treated as described in Experiment 2 except that they were not bled. After 6 days of NWR in LDLD 6:5:3:10, hamsters remained in their home cages for 11 days on the same LDLD cycle described above. Two hamsters with no prior NWR exposure (controls from Experiment 2) were exposed to identical light conditions but were not transferred to novel wheels.

Analyses of activity onsets were performed using data from the last 7 days of exposure to LD 14:10 prior to NWR and the first 7 days of continuous home cage exposure to LDLD 6:5:3:10 after NWR.

Statistical tests (all two-tailed where applicable) were performed with Statview 5.0 software (SAS Institute, Cary, NC, USA).

RESULTS

Experiment 1

Hamsters transferred to novel wheels exhibited robust wheel-running (mean = 8186 ± 160 revolutions/5 h, range = 6817-9210, $n = 20$), with no significant change in amount of wheel running over the 11 days of the experiment ($p > 0.70$, repeated measures ANOVA). When measured on Day 2 of NWR, the number of wheel revolutions varied over time ($p < 0.001$), with significant monotonic increases ($p < 0.05$) over the first 4 h and a decrease from the 4th to the 5th hour ($p < 0.05$).

All 20 hamsters showed a marked reorganization of nighttime activity during NWR characterized by progressive delays in the onset of home cage activity

(Figs. 1, 2). In 2 hamsters (e.g., Fig. 1B), activity onsets delayed so far as to eliminate all nighttime activity on the last 1 to 2 days of NWR. When left in the home cage in the LDLD cycle, 19 out of 20 hamsters showed spontaneous activity in the afternoon dark period, and 17 out of 20 hamsters showed activity in both the afternoon and nighttime scotophases (Fig. 1). The 1 hamster that did not run in the afternoon dark phase was exceptional in having the smallest delay of nighttime activity onset. On each of the final 2 days when hamsters remained in the home cage on the LDLD cycle, a disproportionate amount of running activity occurred in the afternoon scotophase (65% ± 5%, 72% ± 5%, respectively, $n = 20$).

Experiment 2

As in Experiment 1, NWR was observed to be robust in the entire cohort of animals tested (mean = 8153 ± 220 revolutions/5 h, range = 6,127-10,044, $n = 19$). After 7 days in novel wheels, nighttime activity onset was delayed approximately midway through the scotophase (Figs. 2, 3).

Upon release into DD, 8 out of 9 hamsters showed two distinct (i.e., split) activity components roughly coincident with the prior time of nighttime running and novel wheel exposure, respectively (Fig. 3). In DD, nighttime activity onsets occurred progressively later in the first 3 days of DD (Table 1), whereas the afternoon component was neither markedly advanced nor delayed. Thus, the phase angle between components (Ψ_{n-a}) and interval of inactivity initially separating the nighttime and afternoon bouts in DD rapidly diminished. In the first 3 days of DD, the two activity bouts contained comparable amounts of activity, and bouts were characterized by short αs. A redistribution of activity from the afternoon activity component to the nighttime component was commonly seen on the 3rd to 5th day in DD (Fig. 3). The free-running rhythm derived from onsets of the nighttime component shortened significantly after 7 days in DD (Table 1, Fig. 3). A comparable analysis of the afternoon component was not undertaken, because it seldom remained distinct for more than 3 days in DD. A bimodal activity pattern persisted beyond the fifth day of DD, after the disappearance of a robust, clearly distinguishable afternoon component. However, this bimodality appeared to be indistinguishable from that characteristic of unsplit hamsters with comparable α in DD. In other words, by this time the bimodal pattern does not suggest persistent splitting. A quantitative description of nighttime and afternoon activity components in DD is presented in Table 1.

Control animals exposed to identical LDLD conditions showed no reorganization of nighttime locomotor activity patterns or splitting in DD (data not shown).

Experiment 3

As in Experiments 1 and 2, transfer to novel wheels elicited running in the entire sample of experimental hamsters (mean = 7631 ± 268 revolutions per 5 h; range = 4036-9197, $n = 20$), with no significant changes in activity over the 6 days of NWR. Running induced progressive delays in nighttime activity onset. After 6 days of NWR, nighttime activity onset occurred approximately 6 h after lights-out. Compared to substantial further delays observed in identical light conditions in Experiment 1, discontinuation of NWR after 6 days largely prevented further delays in nighttime activity onset (Figs. 2, 4).

Hamsters (18 out of 20) exposed to NWR adopted similar novel entrainment patterns in the home cage under LDLD: Locomotor activity was distributed into two roughly equal components corresponding to the two daily scotophases (i.e., splitting occurred; Fig. 4). Of the 2 nonsplitters, 1 showed the least amount of activity in the novel wheels (4036 rev/5 h) whereas the other showed typical activity levels (7820 rev/5 h). Neither of the 2 control hamsters exposed to this same LDLD cycle, without NWR, adopted this entrainment pattern (data not shown). This split pattern of locomotor activity was sustained for a minimum of 7 days in all 18 animals and for the duration of the experiment (11 days) for 15 of these hamsters.

Quality of entrainment was assessed by examining whether the slope of the best-fitting regression line through activity onsets differed significantly from 24 h ($p < 0.05$). Under baseline entrainment conditions, all but 2 animals yielded regression lines not significantly different from 24 h, indicating that they were well entrained by the 24-h LD cycle. Slopes of these 2 hamsters, moreover, deviated only slightly from 24 h (0.04 and 0.05 h/day, respectively). Likewise, after NWR the afternoon activity component of split hamsters was well entrained under LDLD with only 3 out of 18 split hamsters producing activity onsets with slopes significantly different from 24 h. Activity onsets were less well entrained for the nighttime component of the split rhythms. The majority of hamsters exhibited τ^*s significantly greater than 24 h. Only 3 hamsters had

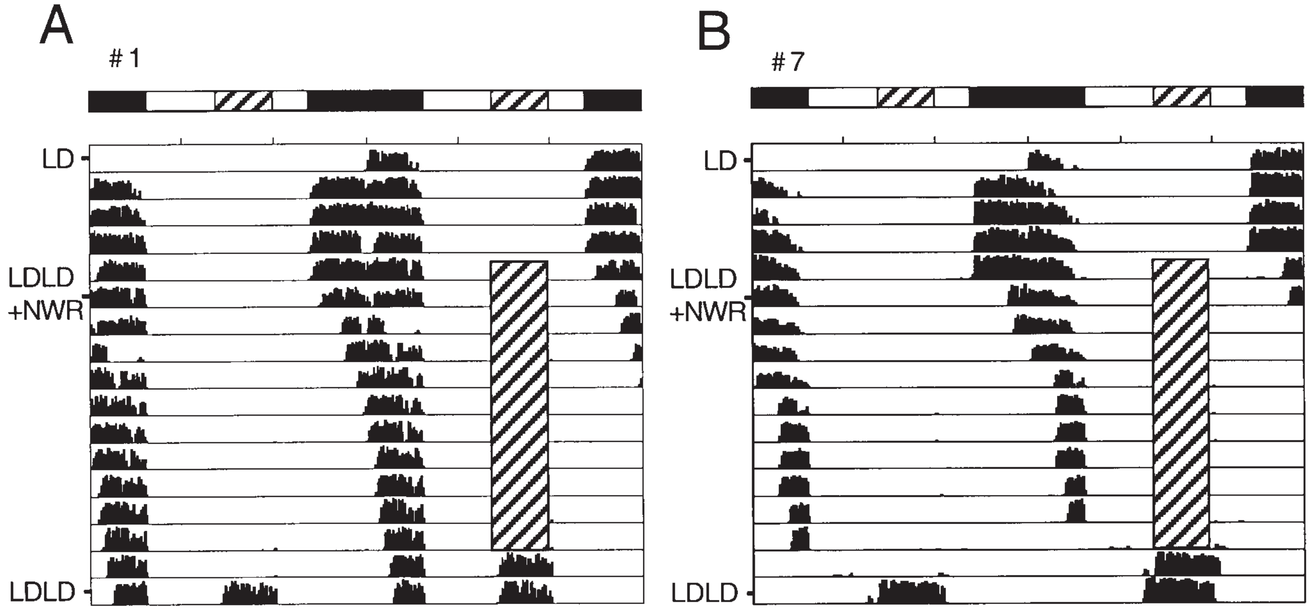

Figure 1. Representative double-plotted actograms of hamsters initially entrained to LD 14:10 and later exposed to daily afternoon novel wheel running (NWR). Data are unclipped and scaled between zero and the hamster's maximum activity count in the interval depicted. Dark rectangles above actograms represent the 10-h scotophase maintained throughout the experiment. Hatched rectangles above and on the right side of actograms represent NWR treatment paired with darkness. This second daily scotophase was maintained for 2 final days when hamsters remained in their home cage.

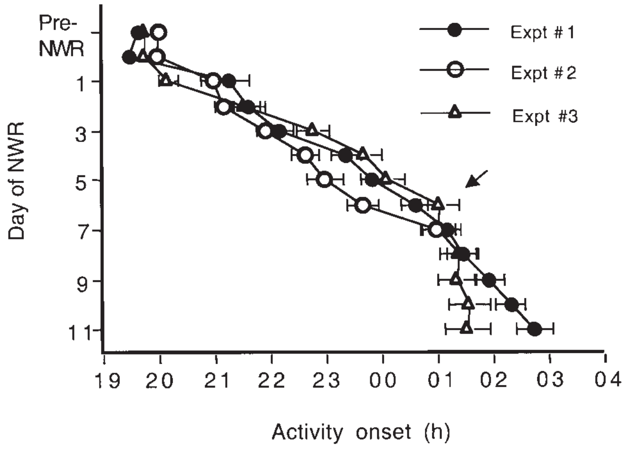

Figure 2. Mean ± SEM activity onsets (clock hour) of hamsters (n = 18-20) exposed to successive days of novel wheel running (NWR) in Experiments 1-3. Onsets for 2 days prior to NWR are designated "Pre-NWR." Hamsters were exposed to 11, 7, and 6 days of NWR in Experiments 1-3, respectively. In Experiment 3, activity onsets for 5 days after NWR was discontinued are plotted for comparison with values in Experiment 1. The arrow indicates when the Experiment 3 hamsters remained thereafter in the home cage without further NWR.

τ^*s not significantly different from 24 h. Finally, quality of entrainment was further assessed by quantifying the sum of squared residuals of actual onsets from the best-fit regression line. Variability of both split components was greater than for the baseline condition (Table 2; $p < 0.001$), but the two components did not differ from one another ($p > 0.05$).

Phase angle to lights-off ($\psi_{lights\text{-}off}$) differed significantly from the unsplit to the split state and between the two split components (Table 2). $\psi_{lights\text{-}off}$ was significantly less negative in the baseline condition prior to NWR than in either the afternoon ($p < 0.05$) or nighttime ($p < 0.001$) activity components in LDLD. In the split condition, $\psi_{lights\text{-}off}$ was far more negative for the nighttime activity component than for the afternoon component. Relative to lights-on ($\psi_{lights\text{-}on}$), the phase angle of the afternoon activity component was greater than that of the nighttime activity component ($p < 0.05$; Table 2). Phase angle of the two split components relative to each other (ψ_{n-a}) varied from 8.40 to 12.24 h (mean = 10.5 ± 0.22 h). Total activity was nearly equally distributed between nighttime (55% ± 2%) and afternoon (45% ± 2%) scotophases.

DISCUSSION

In three separate experiments, daily NWR markedly reorganized the locomotor activity rhythms of male Syrian hamsters maintained in an LD cycle. Nighttime activity onset was progressively delayed with subsequent days of NWR: Whereas nighttime

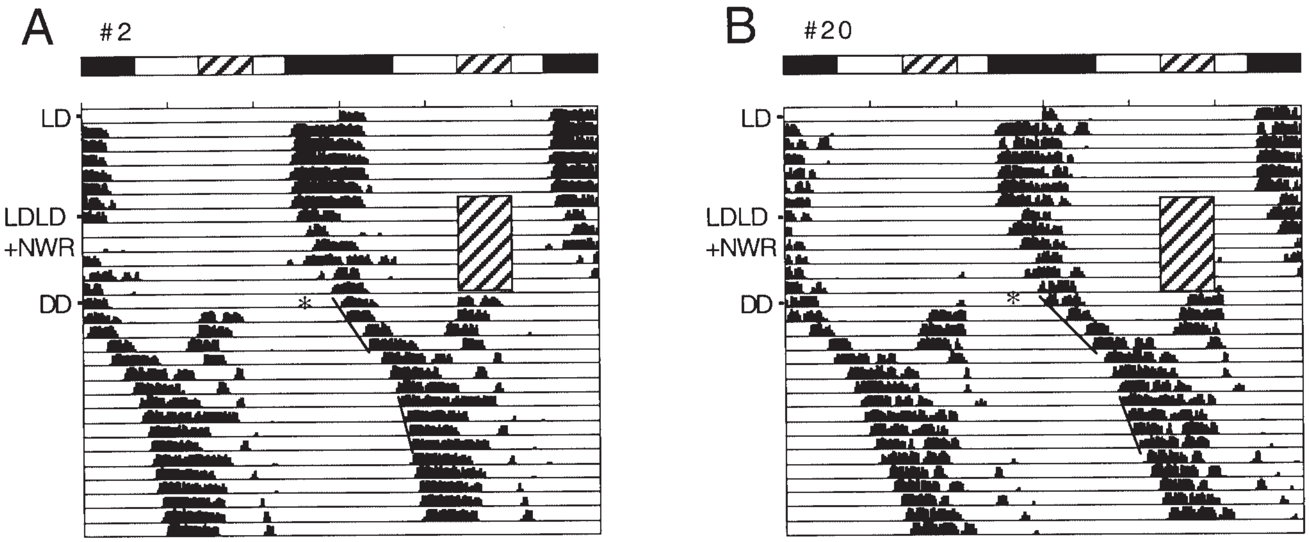

Figure 3. Representative actograms of hamsters from Experiment 2. The asterisk on the actogram indicates beginning of exposure to constant darkness (DD). Slanted lines on right side of the actogram represent least-squares regression lines for nighttime activity onsets on Days 1-4 and 8-11 of exposure to DD. Other conventions as in Figure 1. NWR = novel wheel running.

Table 1. Circadian rhythm patterns of split hamsters ($n = 8$) transferred to DD in Experiment 2. Noted are significant differences over time (repeated measures ANOVA, two-tailed).

	Day 1	*Day 2*	*Day 3*	
Phase angle between components (Ψ_{n-a})(h)	11.6 ± 0.3	10.5 ± 0.3	9.4 ± 0.5	$p < 0.005$
Inactive interval (h)	8.9 ± 0.4	7.8 ± 0.5	5.9 ± 0.5	$p < 0.0001$
Nighttime bout				
Activity onset (h)	1.02 ± 0.18	1.92 ± 0.20	3.21 ± 0.25	$p < 0.0001$
Bout duration (α)(h)	2.70 ± 0.18	2.68 ± 0.33	3.45 ± 0.82	ns
Wheel revolutions	749 ± 61	913 ± 124	1190 ± 274	ns
Afternoon bout				
Activity onset (h)	12.60 ± 0.34	12.40 ± 0.36	12.56 ± 0.52	ns
Bout duration (α)(h)	2.68 ± 0.40	3.25 ± 0.42	2.55 ± 0.45	ns
Wheel revolutions	778 ± 110	1031 ± 132	851 ± 135	$p < 0.01$
	days 1 - 4	days 8 - 11		
τ(h)	25.09 ± 0.15	24.58 ± 0.06		$p < 0.01$

activity disappeared entirely in some hamsters after 11 days of NWR, more modest delays were observed after 6 to 7 days of NWR. This latter condition was associated with distinctly split activity rhythms that rejoined after several days of DD. Perpetuation of the LDLD cycle, however, allowed the split rhythms to be sustained for at least an additional 11 days in the home cage. In the absence of NWR, exposure to the LDLD cycle had no marked effect on nighttime locomotor activity rhythms and yielded no evidence of splitting. As suggested previously (Mrosovsky and Janik, 1993), afternoon NWR phase-shifts some component circadian oscillators, which thereafter give rise to the expression of a new activity bout in the afternoon dark period. Subsequently, when the system is released into DD, the two bouts fuse or rejoin under the influence of strong oscillator interactions, but alternatively may be effectively entrained by an LDLD cycle. NWR can therefore override typical entrainment patterns established in an LDLD cycle and reorganize activity into a second stable configuration.

It is not clear why others have failed to replicate the induction of splitting with afternoon NWR (Sinclair and Mistlberger, 1997) and why NWR induced larger phase-delays of home cage activity onset in this study than in others (Mrosovsky and Janik, 1993). We used the same hamster supplier as the original report, in contrast to the study with largely negative effects.

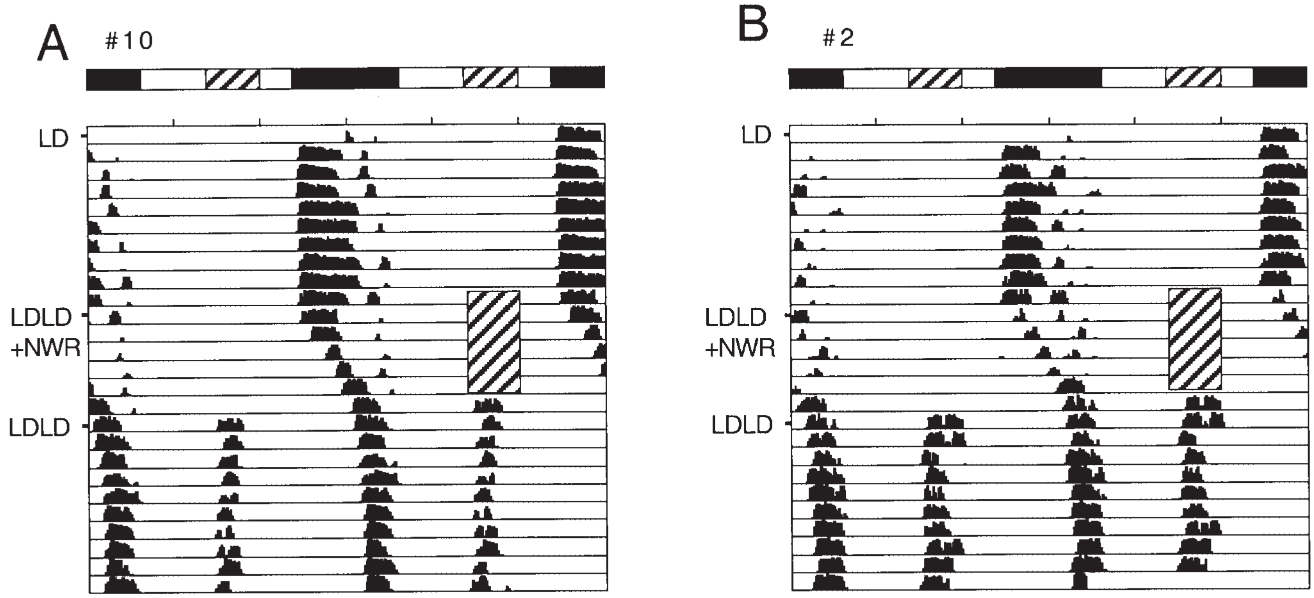

Figure 4. Representative actograms of hamsters from Experiment 3. Conventions as in Figures 1 and 3. NWR = novel wheel running.

Table 2. Entrainment parameters of hamsters expressed in baseline LD 14:10 and following splitting under the experimental LDLD 6:5:3:10 cycle of Experiment 3.

	Baseline	*Nighttime*	*Afternoon*	N
τ^* (h)	24.00 ± 0.01	24.16 ± 0.04	23.99 ± 0.02	18
SS residuals	1.49×10^{-4}	7.90×10^{-4}	7.65×10^{-4}	18
$\Psi_{lights\text{-}off}$ (h)	–0.83 ± 0.02	–6.79 ± 0.22	–1.26 ± 0.05	18
$\Psi_{lights\text{-}on}$ (h)	9.17 ± 0.02	3.21 ± 0.22	3.74 ± 0.05	18

Minor differences in intrinsic periods, propensity to run in novel wheels, or oscillator coupling may distinguish splitting and nonsplitting strains. We also used longer exposures to NWR (5 h vs. 3 h) than used in previous studies. Notably, running in novel wheels was most intense during the 4th hour of exposure. Regardless of differences between studies, the significance of this experimental paradigm is as a probe of specific aspects of oscillator function, which likely differ quantitatively rather than qualitatively among different hamster strains and experimental conditions.

Various formal mechanisms may account for the progressive delays in nighttime activity onset during successive days of NWR. A more negative phase angle of entrainment as was obtained in each experiment can result from a lengthening of τ. Alternatively, each day of NWR may induce a single phase-delay in activity onset without lengthening τ. In prior studies employing a single bout of NWR in early subjective afternoon of hamsters in DD, however, activity onset was advanced rather than delayed, and τ was lengthened (Mrosovsky, 1993; Weisgerber et al., 1997). Moreover, at the conclusion of NWR in the present study, the nighttime activity component free-ran in DD with a long τ. Together, these results suggest an enduring effect on τ as opposed to a transient (e.g., phase shift) effect of NWR on the circadian pacemaker.

Two factors may dictate the pattern of rejoining observed in DD, which in all cases was achieved via reduction of the inactive interval following nighttime activity and preceding afternoon activity. First, independent of any oscillator interactions, the two components may have different intrinsic free-running periods, which would favor rejoining. The large negative phase angle of the nighttime component and the relatively small negative value for the afternoon component suggest free-running periods, which are >24 and <24 h for the nighttime and afternoon components, respectively. Alternatively, coupling interactions between oscillator components may favor the observed pattern of rejoining regardless of the periods of the free-running rhythms. That is, the split state may be intrinsically unstable, and oscillators may interact in DD to establish a limited range of phase angles with respect to each other. Oscillator interactions have been invoked to understand the limited decompression of α, which may be obtained in DD in unsplit hamsters. The pattern exhibited in this experiment is consistent with oscillators recoupling by the shortest possible route (i.e., reducing the shorter of the two respective phase angles, Ψ_{n-a} versus Ψ_{a-n} between them), although this proposition cannot be evaluated against alternatives with the present data set. A role of oscillator interactions is further suggested by the marked change in τ measured from the nighttime component after several days in DD, when splitting is presumably ended.

The results of Experiment 3 complement those of Boulos and Morin (1985) who, with daily dark pulses, entrained activity rhythms split by LL. In that study, one component roughly coincided with a daily 2-h dark phase, while the second activity component persisted 8 to 12 h out of phase with the dark-entrained component. In the current study, it appears that the split nighttime activity component, which free-runs in DD with $\tau > 24$ h, may be entrained solely by the phase-advancing effects of light onset at ZT 22. In contrast, the split afternoon activity component may be entrained by either phase-delaying effects of light prior to lights-off at ZT 4, by phase-advancing effects of lights-on at ZT 9, or by both. In hamsters split by LL, each component of the activity rhythm expresses a PRC to dark pulses with defined regions of delays and advances (Boulos and Rusak, 1982).

How does NWR split circadian activity rhythms? Convergent evidence from cellular, physiological, behavioral, and mathematical paradigms (e.g., Illnerova, 1991; Liu et al., 1997; Pittendrigh and Daan, 1976; Enright, 1980) points to the following model of the circadian pacemaker: Overt circadian rhythms reflect the output of multiple circadian oscillators that constitute a coupled dual oscillatory system, which may be functionally described in terms of evening and morning oscillators (Fig. 5A). A functional evening oscillator results from the coupling of oscillators with relatively short τs, and as such, its overall τ is < 24 h, whereas a functional morning oscillator is derived from coupling of longer period constituent oscillators with $\tau > 24$ h. As one effect of NWR at ZT 4 appears to be a marked lengthening of the period of the nighttime activity component (Mrosovsky, 1993; Weisgerber et al., 1997), and because NWR delayed nighttime activity onset (present studies), we hypothesize that early afternoon NWR preferentially lengthens the period of the oscillators underlying nighttime activity, perhaps by uncoupling some of the short-period component "evening" oscillators from the coupled oscillator network that generates normal nighttime activity (Fig. 5A). In DD, the larger coupled system might therefore free-run under the influence of its remaining coupled constituent oscillators, of which those with longer intrinsic τs predominate. Under entraining LD conditions, one would expect a more negative phase angle as a result of the oscillator's lengthened τ. Whether NWR selectively uncouples short-period oscillators because of a particular anatomical relationship (e.g., such oscillators receive neuropeptide Y projections) or a temporal relationship (e.g., a particular phase angle between NWR and short-period oscillators) is entirely unknown.

Additionally, NWR apparently phase-shifts constituent oscillators just as it phase-shifts without overtly splitting the pacemaker in two other paradigms: After an 8-h phase-advance of the LD cycle, hamsters running in novel wheels during the new ZT 13-16 re-entrained within one to two cycles, whereas nonrunning controls required several days (Mrosovsky and Salmon, 1987). NWR of sufficient duration beginning at ZT 5, moreover, induced rapid phase shifts in excess of 8 h in some hamsters transferred to DD (Gannon and Rea, 1995). The present paradigm likewise induces phase shifts, albeit of only a fraction of the oscillators formerly generating the nighttime activity. The present data strongly suggest that successive days of NWR recruit cohorts of oscillators to express their subjective night in the afternoon scotophase. After 6 to 7 days of NWR, activity is nearly equally divided between nighttime and afternoon scotophases, whereas the afternoon scotophase contains disproportionate activity (and in some cases all) after 11 days of NWR. We suggest that a single day of NWR produces a large phase shift of a small fraction of component oscillators. With additional days of NWR, a threshold fraction of oscillators may be phase-shifted to generate an activity component in the afternoon scotophase. The progressive delays in nighttime activity onset are consistent with this model.

In contrast to other paradigms used (Gannon and Rea, 1995; Mrosovsky and Salmon, 1987), the presence of an LDLD cycle with a short (5 h) second scotophase may prevent the entire complement of oscillators from being phase-shifted to express subjective night in the afternoon. Moreover, in the absence of further NWR, the light pulses that bracket the afternoon scotophase impede the recoupling of constituent oscillators back into the unsplit state (Experiment 3), which so readily occurs in DD. Notably, when the intervening light intervals were very short as in skeleton photoperiods, daily NWR from ZT 5 to ZT 8 induced complete inversion of activity rhythms to what was previously subjective day (Sinclair and Mistlberger, 1997). Similarly, 11 days of NWR apparently also shifted the entire oscillatory system in a few hamsters of Experiment 1. Thus, these two factors—a titratable shifting of oscilla-

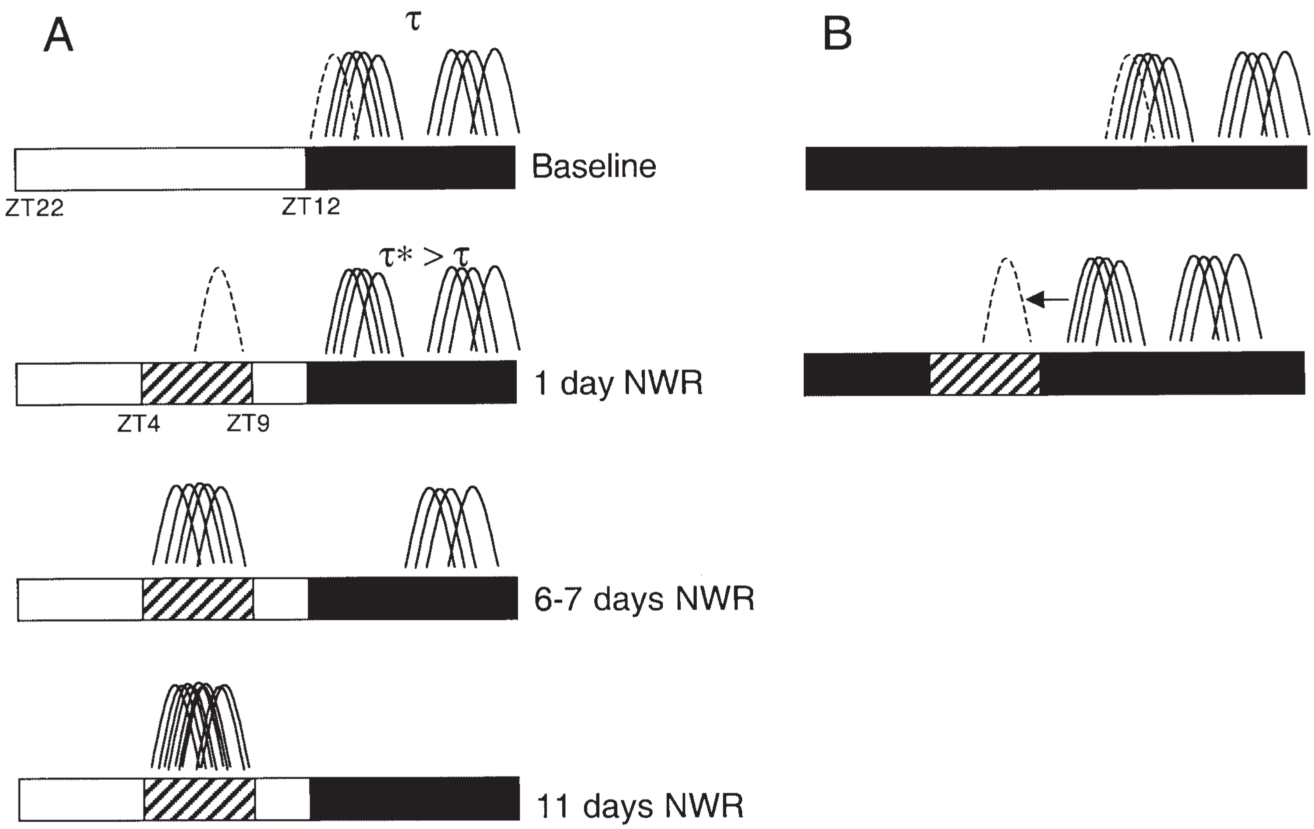

Figure 5. Formal model of multioscillator basis of novel wheel running (NWR) effects on circadian rhythms. In all panels, the inverted U shape represents subjective night of hypothetical individual circadian oscillators. For clarity, the remaining phases of each oscillator are not depicted. In *A*, the integrated rhythm reflects a distribution through the scotophase of the subjective nights of individual oscillators. Those with short and long periods express subjective night early and late in the scotophase, respectively. The oscillator denoted with a dashed line will be phase-shifted by NWR. After a single day of NWR from ZT 4 to ZT 9, oscillators with short periods undergo large phase shifts to roughly the same circadian phase as NWR. Light pulses bracketing NWR preclude oscillator recoupling for reasons that are not yet clear. Subsequent activity onset is delayed, and τ is lengthened (τ* > τ). Additional days of NWR phase-shift additional cohorts of oscillators such that approximately half are phase-shifted after 6 to 7 days but all or nearly all are shifted after 11 or more days. *B*. Application of the splitting model to understand effect of a single day of NWR applied in DD. In the absence of a light pulse following NWR, the phase-shifted oscillator pulls (advances) the nighttime component that follows. Until full recoupling is achieved, the nighttime activity component may continue to express a lengthened τ.

tors and countering of oscillator tendencies to rejoin—may facilitate induction and maintenance of stable, split activity rhythms under an LDLD cycle and 1 or more days of NWR.

Beyond the significance of NWR-induced splitting noted previously (Mrosovsky and Janik, 1993), the demonstration that some oscillatory components can be dissociated from others and rephased with respect to the nighttime activity component may provide insight into the mechanism of phase-shifting effects of NWR, or indeed, of any photic or nonphotic zeitgeber. For example, a single bout of NWR around ZT 5 produces large phase advances and lengthening of τ in subsequent DD (Mrosovsky, 1991; Reebs and Mrosovsky, 1989a, 1989b; Weisgerber et al., 1997). A single bout of NWR is not sufficient to induce measurable splitting under LDLD conditions (MR Gorman, unpublished observations), but if it selectively phase-shifts a small fraction of circadian oscillators (Fig. 5B), overt circadian rhythms might be altered as a consequence of recoupling dynamics among constituent oscillators. Unfortunately, little is known about these processes, except that darkness favors recoupling in LL-split hamsters (Earnest and Turek, 1982) and intervening light pulses appear to minimize recoupling in NWR-induced splitting (Experiment 3). Actograms of NWR-split hamsters suggest that recoupling may be accompanied by abrupt changes in phase, with the rejoining activity bout typically expressed in an intermediate phase between the former two components (Mrosovsky and Janik, 1993). If no light follows a bout of NWR, strong oscillator interactions may result in rapid recoupling via reduction of the shorter phase angle between component oscillators. This would advance rather than delay the main activity onset (Fig. 5B). Consistent with this general model, light pulses shortly after a single day of NWR, such as those that impeded oscillator recoupling in the present study, greatly attenuated the phase-advancing effects of NWR (Mrosovsky, 1991).

An understanding of oscillator-oscillator interactions has lagged behind our knowledge of other features of circadian rhythms, although physiological data suggest that such interactions must be central to an understanding of the pacemaker. Only a fraction of SCN cells, for instance, receive direct retinal or IGL projections, and yet both of these pathways are capable of phase-shifting, presumably, the entire SCN. Any zeitgeber, therefore, first shifts a subpopulation of SCN cells that receives direct projections from the time-giving entrainment mechanism. These cells in turn interact with the greater complement of SCN cells to arrive at a steady-state phase shift. Under routine conditions, this selective shifting and oscillator interaction may happen in a cycle or even more rapidly. The use of LDLD cycles in the present paradigm, in contrast, facilitates a temporal dissociation of these processes by impeding the recoupling process.

ACKNOWLEDGMENTS

We thank Jim Donner for excellent animal care and Jeff Elliott for helpful comments on an earlier draft of this manuscript. This research was supported by NHLBI grant HL61667 to TML and NICHD-48640 to MRG.

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