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What is This?

Entrainment of 2 Subjective Nights by Daily Light:Dark:Light:Dark Cycles in 3 Rodent Species

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Abstract   Recent work with exotic 24-h light:dark:light:dark (LDLD) cycles indicates surprising flexibility in the entrainment patterns of Syrian hamsters. Following exposure to an LDLD cycle, hamsters may adopt a form of rhythm splitting in which markers of subjective night (e.g., activity, melatonin) are expressed in each of the twice daily scotophases. This pattern contrasts markedly with that of conventionally entrained hamsters in which markers of subjective night are expressed once daily in only 1 of the 2 dark periods. The "split" entrainment pattern was examined further here in Syrian and Siberian hamsters and in mice exposed to LDLD 7:5:7:5, a condition that reliably induces split activity rhythms in all 3 species. The phase angle of entrainment and activity duration were generally similar comparing the 2 daily activity bouts in each species. The stability of this split entrainment state was assessed by deletions of photophases on individual days, by exposure to skeleton photoperiods, and by transfer to constant darkness. As in Syrian hamsters, the one-time substitution of darkness for one 7-h photophase did not grossly alter activity patterns of Siberian hamsters but acutely disrupted the split rhythms of mice. Skeleton light pulses of progressively shorter duration did not significantly alter split entrainment patterns of either Syrian or Siberian hamsters. Both species continued to exhibit stable entrainment with activity expressed in alternate scotophases of an LD 1:5 cycle presented 4 times daily. In contrast, the split activity rhythms of mice were not maintained under skeleton pulses. In constant darkness, rhythms of Siberian hamsters remained distinctly split for a minimum of 2 cycles. Split entrainment to these novel LDLD and 4-pulse skeleton lighting regimes demonstrates a marked degree of plasticity common to the circadian systems of several rodent species and identifies novel entrainment patterns that may be reliably elicited with simple environmental manipulations. Inter- and intraspecific differences in the stability of split activity rhythms likely reflect differences in coupling interactions between the component circadian oscillators, which, adopting separate phase relations to these novel LD cycles, yield a split entrainment pattern.

Key words   splitting, coupling, photoperiod, multiple circadian oscillators, wheel running, entrainment, light

Many behavioral and physiological processes vary on a daily basis under the influence of a neural oscillator in the mammalian suprachiasmatic nuclei (SCN) of the anterior hypothalamus. In constant darkness, the SCN drive an alternation between coordinated states representing "subjective night" and "subjective

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day," with a free-running period generally not exactly 24 h. For nocturnal rodents, subjective night is marked by elevated activity levels and pineal melatonin secretion and by sensitivity to the phase-shifting and clock-gene altering effects of light. Subjective day, in contrast, is characterized by relative inactivity, low melatonin secretion, and insensitivity to phase-resetting by light (Elliott and Tamarkin, 1994; Illnerova et al., 2000; Pittendrigh, 1988; Weaver, 1998). Entrainment of the circadian pacemaker to the 24-h sidereal day by environmental light-dark cycles is facilitated by a circadian-gated rhythm in light responsiveness that is described by a phase response curve (PRC) (Johnson et al., 2003). In nocturnal rodents, exposure to relatively brief light pulses at dawn and dusk in a 2-pulse skeleton photoperiod is sufficient for entrainment that is often nearly indistinguishable from that of the corresponding full photoperiod (Pittendrigh and Daan, 1976).

Until recently, the coordinated circadian alternation between daytime and nighttime physiologies in mammals was known to break down only in lighting conditions characterized by the absence of entrainment. Following prolonged exposure to constant light, behavioral and physiological rhythms of hamsters may bifurcate into 2 components that initially express different periods. They later free-run with a common period but are separated in phase by approximately 12 h (180° antiphase) (Pickard et al., 1984; Pittendrigh and Daan, 1976; Swann and Turek, 1985; Turek et al., 1982). This light-induced "splitting" is associated with antiphase oscillations of the left and right SCN (de la Iglesia et al., 2000). Similarly, the diurnal tree shrew, *Tupaia belangeri*, may exhibit a formally similar splitting phenomenon in constant dim illumination or darkness (Hoffmann, 1971). An internal desynchronization of circadian rhythms, moreover, may occur in humans and nonhuman primates maintained in constant conditions or on self-selected lighting (Aschoff, 1965; Czeisler et al., 1980; Sulzman et al., 1979; Wever, 1979). Whereas core body temperature commonly free-runs with a period near 24 h, the length of the rest-activity cycle and other associated rhythms may increase substantially so that the 2 sets of rhythms free-run with different periods. Both splitting of animal rhythms under constant light and internal desynchronization in humans suggest that the circadian system comprises multiple oscillators that are normally coupled to generate a coordinated and integrated circadian output.

Recently, we have devised conditions for dissociating component circadian oscillators and entraining them to a light:dark:light:dark (LDLD) cycle. Initially, scheduled repeated access to a novel running wheel was used to induce progressive changes in locomotor activity rhythms in Syrian hamsters (Gorman and Lee, 2001; Mrosovsky and Janik, 1993). After 11 days, a majority of hamsters left in their home cages expressed robust wheel-running behavior in each of 2 daily scotophases. Hamsters exposed to identical lighting conditions but not transferred to novel running cages, and hamsters electing not to run in novel cages (i.e., sluggards), entrained with activity confined only to 1 scotophase. The circadian reorganization following novel wheel running (NWR) reflects a global splitting of the circadian system as melatonin secretion as well as light-induced phase shifts and SCN Fos expression were similarly split into 2 daily fractions, 1 associated with each of the twice daily scotophases (Gorman et al., 2001). Recently, this temporal reorganization was shown to extend to Per1 and Per2, which were symmetrically expressed in the 2 SCN nuclei (Edelstein et al., 2003). Thus, both LL-induced and NWR-split hamsters exhibit 2 intervals of subjective day and 2 intervals of subjective night in each 24-h day, but only the former is apparently associated with antiphase oscillations of the left and right SCN. We have subsequently devised methods for more rapidly obtaining a split entrainment pattern in a majority of Syrian hamsters without repeated exposure to novel wheels (Gorman, 2001; Gorman et al., 2003). Briefly, entrained split activity rhythms emerge reliably following transfer of wheel-naive hamsters in LD 14:10 to running cages at the onset of the first scotophase of a new LDLD 7:5:7:5 cycle (Gorman et al., 2003).

For both NWR-induced and LDLD-induced split rhythms, the 2 activity components quickly rejoin in constant darkness under the influence of strong oscillator interactions (Gorman, 2001; Gorman and Lee, 2001). As this re-joining can be prevented indefinitely under an LDLD cycle, the 2 photophases suppress these interactions or produce daily phase-shifts that counter these coupling forces. The present study investigated the entraining actions of the intervening photophases through the use of "skeleton photoperiod" cycles employing 4 light pulses per 24 h. Here, each full 7-h photophase of the splitting LDLD light cycle is replaced with 2 shorter light pulses timed to match the prior D/L (light onset) and L/D (light off-

set) transitions. With very brief light pulses and normal unsplit rhythms, 2-pulse skeleton photoperiods generally mimic the entraining actions of full photophases except for very long full photoperiods where this equivalence breaks down (Pittendrigh and Daan, 1976). Here we demonstrate that 3-h, 2-h, and 1-h light pulse skeleton LD cycles (4 light pulses/day) are sufficient to entrain the split activity rhythms of Syrian hamsters. Analogous split activity patterns in Siberian hamsters and mice are also readily induced by LDLD exposure, but only the former species can be maintained in the split state by 4-pulse skeletons.

METHODS

General

All animals were maintained on corncob bedding with ad libitum access to water and Purina chow (St. Louis, MO). Temperature was 22 ± 2 °C. At all times in each of the 3 experiments described below, each animal cage was illuminated by a proximately located green light emitting diode (LED). This lamp generated a "darkness" scotophase intensity < 0.02 lux at the brightest location in the cage. Throughout the following discussion, we use the terms *full* or *full photoperiod* to refer to photophases of 7 h or more in length even when they occur twice per 24 h as in baseline split inducing LDLD conditions (e.g., LDLD 7:5:7:5). We reserve the use of the term *skeleton* to refer to short light pulses (1-3 h duration; 4 per 24 h) employed to mimic the entraining action of the D/L and L/D transitions of the twice-daily 7-h photophases.

EXPERIMENT #1: SYRIAN HAMSTERS

Male ($n = 15$) and female ($n = 15$) Syrian hamsters (*Mesocricetus auratus*) born in the laboratory from Harlan stock (HsdHan:AURA, Indianapolis, IN) had been transferred at 18-19 weeks of age from LD 14:10 to LDLD 7:5:7:5 using a protocol slightly modified from others previously demonstrated to reliably split activity rhythms in this species (Gorman, 2001; Gorman et al., 2003). These minor protocol variations, which include age and timing of exposure to running wheels, are not the subject of this report but may have contributed to a lower incidence of splitting in the present study. After 4 weeks in LDLD 7:5:7:5, 7 hamsters with split rhythms ($n = 4$ females, $n = 3$ males)

and 4 hamsters with unsplit rhythms ($n = 3$ females, $n = 1$ male) were selected for further study with skeleton light pulses. As illustrated in Figure 1, progressively shorter light pulses were used as skeletons for both of the original 7-h photophases (i.e., 7-h photophases were replaced by LDL 3:1:3 for 2 weeks, LDL 2:3:2 for an additional 2 weeks, and finally LDL 1:5:1 for 6 weeks). The final skeleton photoperiod yields an LD 1:5 cycle repeated 4 times daily. Throughout, hamsters were held in polypropylene wheel-running cages ($27 \times 20 \times 15$ cm; 17 cm diam. wheel) kept within individual, light-tight, ventilated chambers with a photophase intensity of ~150 lux provided in each chamber by a single 4 W fluorescent bulb centered over the wheel cage.

EXPERIMENT #2: SIBERIAN HAMSTERS

Colony-bred male Siberian hamsters (*Phodopus sungorus*; $n = 16$), 6-7 weeks of age, were transferred from LD 14:10 at ZT 2 (lights off defined as ZT 12) to cylindrical wheel-running cages (20 cm diam. × 25 cm high; 15 cm diam. wheel), which began the 1st scotophase of a new LDLD 7:5:7:5 photocycle. Cages were kept in chambers housing 8 animals each, with one 15 W fluorescent lamp per 2 hamsters generating an illuminance level of ~150 lux. For half of the hamsters, several layers of shade cloth were used to reduce photophase intensity to ~30 lux. After 4 weeks of LDLD 7:5:7:5 exposure, all animals experienced a "probe day" during which a single 7-h evening photophase was replaced with darkness. Three days later, darkness replaced a single morning photophase. Beginning 9 days later, skeleton photoperiods as described in Experiment #1 replaced each 7-h photophase. After 6 weeks of the final LD 1:5 photocycle, hamsters were released into constant darkness beginning during either the daytime or the nighttime scotophase. Two weeks prior, shade cloth had been removed from the fluorescent lamps to equalize photophase intensity for all hamsters.

EXPERIMENT #3: MICE

Male mice (*Mus musculus*; CH3B6, $n = 8$ and C57BL/6, $n = 8$; Harlan), 5-6 weeks old, were transferred from LD 12:12 (lights on 0300 PST) at ZT 5 (0800 PST) in the same caging as described in Experiment #2. Again, this began a daytime scotophase of LDLD

7:5:7:5. Half of the mice of each genotype were exposed to the standard photophase light intensity (~150 lux) and half to dimmer light (~30 lux). In probe days that began after 4 weeks in LDLD 7:5:7:5, a single morning photophase was replaced with darkness. Three days later, darkness was similarly substituted for a single evening photophase. After an additional 4 weeks, each 7-h photophase was replaced 1st with 3-h skeleton pulses for 2 weeks, and then with 2-h skeleton pulses. A light failure compromised the skeleton photoperiods for 2 mice.

ANALYSES

For all experiments, data were compiled into 6-min bins by DataQuestIII software (Mini Mitter, Bend, OR). Actograms were prepared in ClockLab (Actimetrics, Evanston, IL), and statistical analyses were performed with Statview 5.0 software (SAS Institute, Cary, NC). To characterize the activity patterns, 24-h histograms were prepared by averaging activity levels for each bin over 7-14 days. Working from the middle of each photophase, activity onset for this interval was defined as the 1st of 3 consecutive bins where average activity counts exceeded 5/min. Similarly, activity offset was the last of 3 consecutive bins meeting this criterion, and activity duration was calculated as the difference between these 2 values. As there were no salient group differences between animals exposed to bright (~150 lux) versus moderate (~30 lux) intensity photophases or between mice of different genotypes, these groups were appropriately merged and the associated variables are not considered further.

Rhythms were categorized as split entrained, unsplit entrained, or unentrained. Entrained rhythms expressed a dominant period of 24 h over any given 2-week interval. Animals were classified as split if threshold activity levels were reached in each of two 5-h scotophases for 6 successive bins on 7 days in any given 14-day interval. In practice, there was no ambiguity about whether animals were split or not. For split animals, phase angles of entrainment (activity onset relative to lights off) were calculated for each activity component in relation to the 5-h daytime or nighttime scotophase, respectively. For quantitative analyses, nighttime versus daytime scotophase entrainment parameters were compared using paired t tests and considered significant at $p < 0.05$, uncorrected for multiple comparisons. Changes in activity

onset and duration of each component were evaluated with repeated measures analyses of variance (ANOVA; Statview 5.0; SAS Institute, Cary, NC), with 1 within-subject factor with 4 levels (full and each skeleton photoperiod).

RESULTS

Experiment #1: Syrian Hamsters

The nighttime and daytime activity components of the 7 hamsters with split rhythms were not differentially entrained to their respective scotophases nor did they differ in activity duration. Of these 7 hamsters originally entrained to LDLD 7:5:7:5, 6 continued to entrain through skeleton pulses of progressively shorter duration (Fig. 1 A,B). The 2 activity components showed modest, but statistically significant, differences in the phase angle of entrainment to dark onset when skeleton pulses were 2 h or 1 h (Table 1). Among the 6 hamsters exhibiting persistent entrainment, no circadian measure (activity onset or duration of either component) varied significantly as a function of the full and skeleton photoperiods (Table 1; $p > 0.10$). The activity rhythm of the 7th hamster rejoined during exposure to the 1st (3-h) skeleton light pulses.

The 4 unsplit hamsters entrained to LDLD 7:5:7:5 with evidence of negative masking of activity onset by light (Fig. 1C). Mean activity onset anticipated lights off by 0.6 (± 0.6) h, which differed significantly from the phase angle of entrainment of each of the split (daytime and nighttime) activity components ($p < 0.05$; cf. split data in Table 1). Activity duration of the unsplit rhythm (5.3 ± 0.7 h) was also significantly longer than either the daytime or nighttime component of the 7 split rhythms ($p < 0.05$). Under skeleton photoperiods, activity onset of 2 unsplit hamsters advanced into earlier scotophases, and entrainment was unstable (e.g., Fig. 1C). For 2 other hamsters, however, activity remained confined to a single 5-h scotophase.

No significant sex differences in activity duration and onset were apparent, but the small sample of each sex precluded a rigorous statistical analysis.

Experiment #2: Siberian Hamsters

Of 16 Siberian hamsters, 13 adopted stable split activity patterns in LDLD 7:5:7:5, very similar to those shown for Syrian hamsters (Fig. 2A). Of the remaining
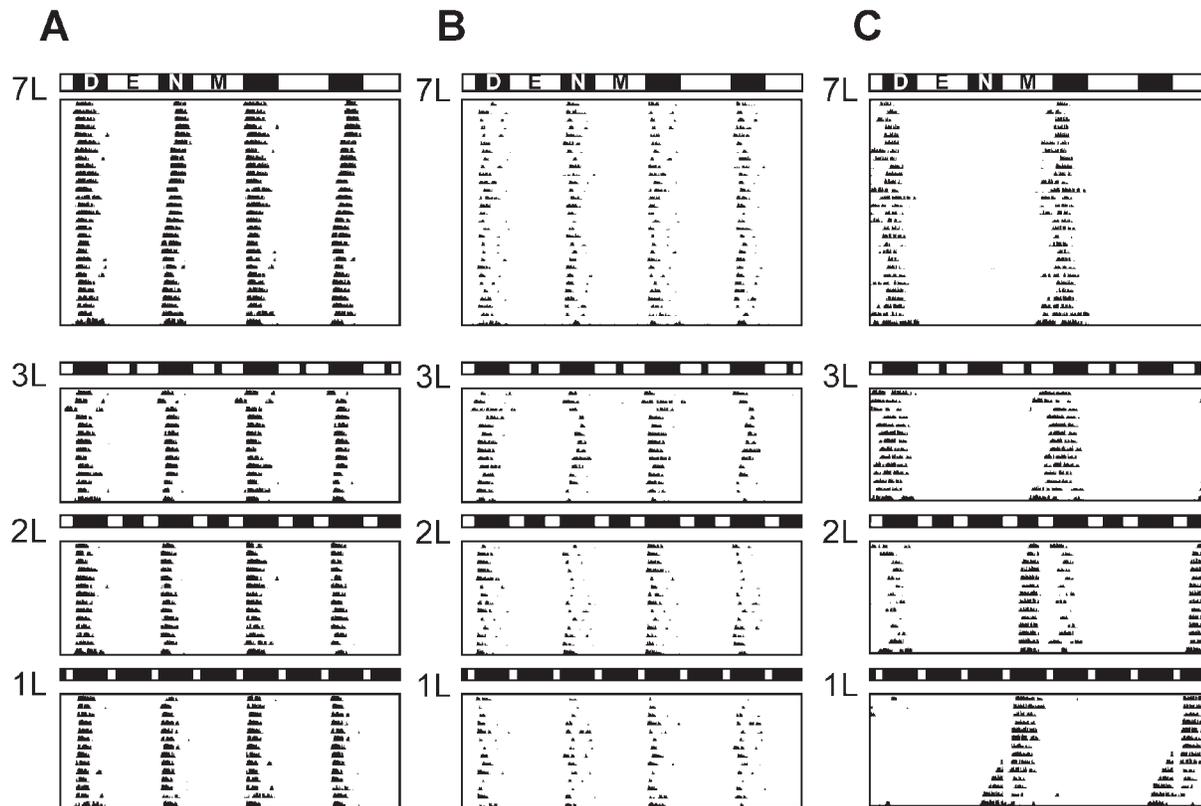
Figure 1. Representative double-plotted actograms of split (A, B) and unsplit (C) male (A) and female (B, C) Syrian hamsters maintained under 24-h light:dark:light:dark cycles (above, LDLD 7:5:7:5) and under corresponding skeleton photoperiods (below). Actograms are scaled from 0 to 150 counts/min. Black and white rectangles above the actograms represent times of dark and light, respectively. At the top of each record, the twice daily 5-h scotophases are designated as D (daytime) and N (nighttime), respectively. These scotophases are separated by 7-h M (morning) and E (evening) photophases. The top section of each record (A, B, C) shows entrainment to the full photoperiod LDLD cycle described above, while the lower 3 panels show responses to replacement of the 7-h photophases with skeleton light pulses of diminishing length (3 h, 2 h, 1 h). See text for further details.

Table 1.   Entrainment parameters (mean ± SEM) of hamsters and mice under light-dark:light-dark cycles and under skeleton photoperiods.

	Phase Angle of Activity Onset (h before lights off)		Night/Day Difference	Activity Duration (h)		Night/Day Difference
	Night	Day	P	Night	Day	P
Syrian Hamsters						
Full LDLD ($n = 7$)	−0.60 ± 0.10	−0.71 ± 0.24		3.69 ± 0.20	3.34 ± 0.22	
3-h skeleton light pulses ($n = 6$)	−0.18 ± 0.21	−0.13 ± 0.31		3.70 ± −0.27	4.10 ± 0.33	
2-h skeleton light pulses ($n = 6$)	−0.57 ± 0.09	−0.37 ± 0.04	< 0.05	3.27 ± 0.29	3.58 ± 0.30	
1-h skeleton light pulses ($n = 6$)	−0.58 ± 0.07	−0.42 ± 0.02	< 0.05	3.40 ± 0.24	3.42 ± 0.24	
Siberian hamsters						
Full LDLD ($n = 13$)	−0.44 ± 0.17	−0.22 ± 0.14		4.71 ± 0.14	4.92 ± 0.14	
3-h skeleton light pulses ($n = 13$)	−0.42 ± 0.15	−0.10 ± 0.17	< 0.05	4.51 ± 0.12	5.09 ± 0.17	< 0.01
2-h skeleton light pulses ($n = 13$)	−0.30 ± 0.11	−0.16 ± 0.14		4.65 ± 0.24	5.03 ± 0.12	
1-h skeleton light pulses ($n = 13$)	−0.45 ± 0.16	−0.14 ± 0.21		4.59 ± 0.12	5.1 ± 0.21	< 0.01
Mice						
Full LDLD ($n = 13$)	−0.13 ± 0.09	0.08 ± 0.13	< 0.01	5.11 ± 0.11	5.01 ± 0.13	
3-h skeleton light pulses ($n = 11$*)	−0.25 ± 0.13	0.29 ± 0.30		4.85 ± 0.21	5.32 ± 0.13	

*Two cases omitted due to light failure.

3 hamsters, 2 never exhibited marked locomotor activity in the daytime scotophase, whereas 1 exhibited a

transiently bimodal rhythm that was subsequently consolidated with activity centered in the daytime
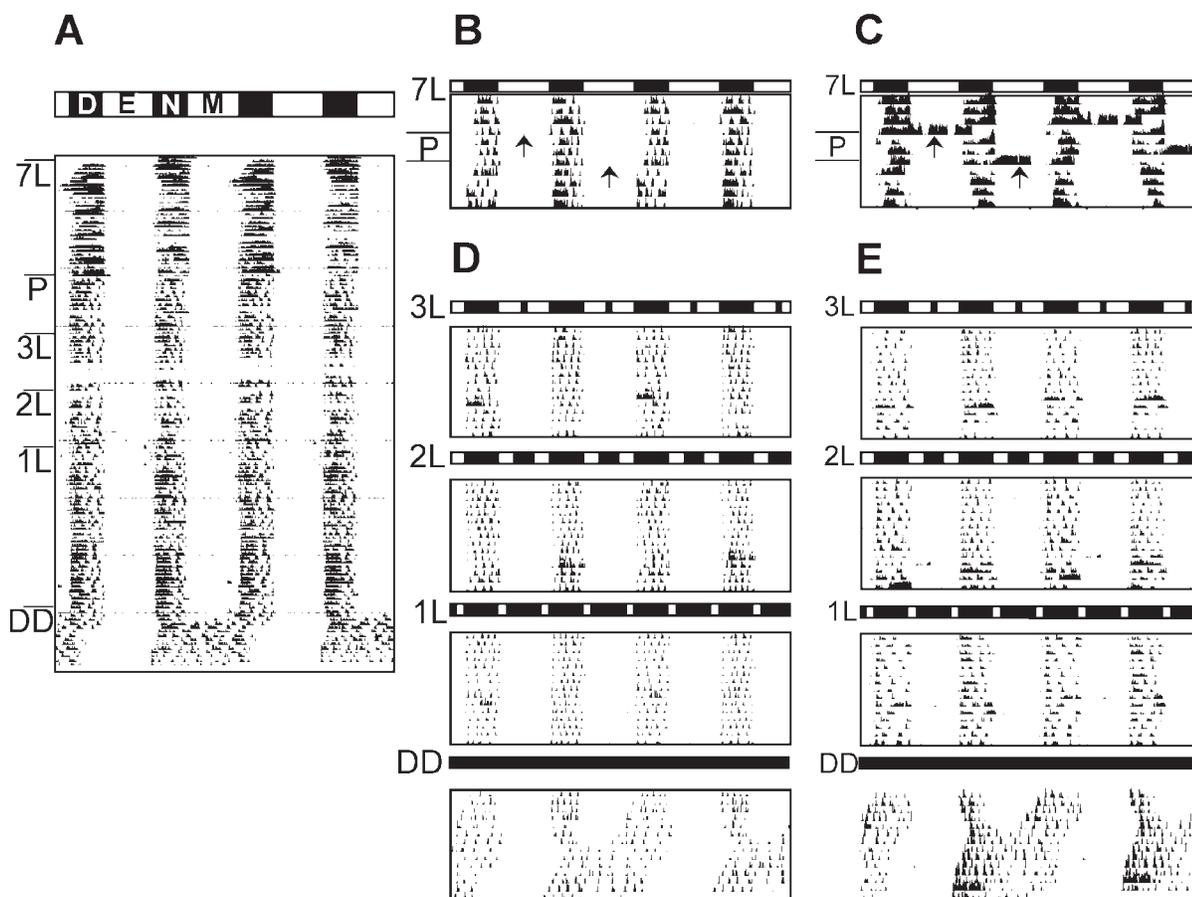
Figure 2. Representative double-plotted actograms of Siberian hamsters with split rhythms in Experiment #2. A complete record (A) begins at the time of transfer from LD 14:10 to the LDLD 7:5:7:5 cycle depicted above the actogram. Two probe days 3 days apart during which the evening or morning photophases were replaced with darkness are noted with a P on the left of the full actogram. The interval just before and after these probes is enlarged for 2 different animals in panels B and C, and the times of the photophase deletions are represented with arrows. After 6 weeks (including probe days), the LDLD cycle was replaced with skeleton photoperiods with 3-h, 2-h, and 1-h pulses, respectively, as noted in A and enlarged in D and E. The bottommost panels illustrate activity in constant darkness (DD), which began during the 1st cycle shown after the daytime or nighttime scotophase (panels D and E, respectively). Other conventions as in Fig. 1.

scotophase (not shown). Phase angles of entrainment did not differ for the daytime and nighttime components (Table 1). On probe days, the typical split hamster exhibited no marked change in the bimodal activity rhythm when the evening or morning photophase was replaced with darkness (Fig. 2B). One of 2 exceptions to this generalization is illustrated in Fig. 2C in which activity persisted into the interval with the deleted photophase. The 2 exceptional hamsters exhibiting large changes in activity offset during these probe days responded similarly to deletions of both the morning and the evening photophases (Fig. 2C).

The split entrainment pattern observed in LDLD 7:5:7:5 was unambiguously maintained in all 13 Siberian hamsters exposed to skeleton photoperiods (Fig. 2 D,E). For both the daytime and nighttime activ-

ity components, neither phase angle of entrainment nor activity duration was significantly altered in full LDLD photoperiods versus any of the skeleton conditions (Table 1; $p > 0.40$ for all tests). Small, but statistically significant differences between daytime and nighttime activity components were noted for phase angle of entrainment and activity duration (Table 1). In constant darkness, the split rhythms remained distinct for a minimum of 2 cycles in all hamsters (with at least 6 h of inactivity between components) and for considerably longer in most cases (Fig. 2 D,E). A single, longer active phase was eventually apparent after 4-7 cycles. In all but 2 cases, the onset and offset of the re-joined rhythm were identifiably continuous with those of the nighttime and daytime activity components, respectively, of the previous split rhythm. This
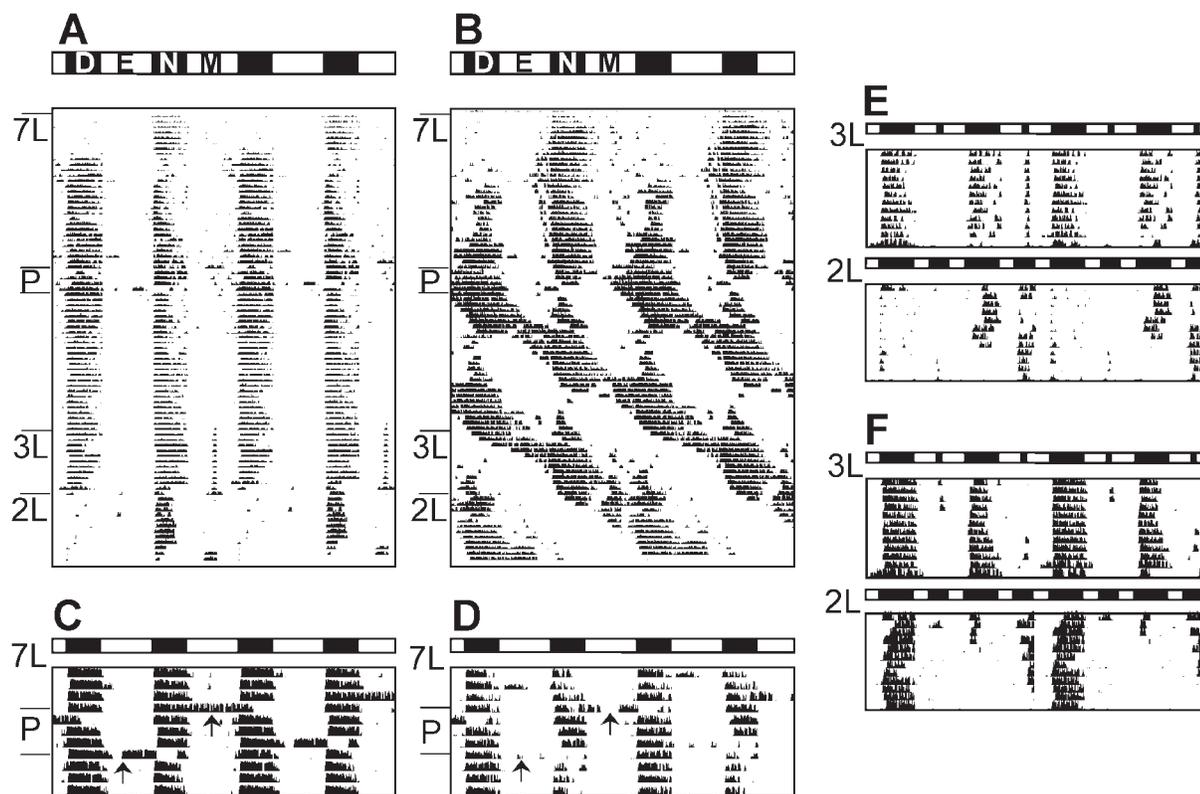
Figure 3. Representative double-plotted actograms of mice with split (A) or unentrained (B) rhythms in Experiment #3. Conventions as in Figs. 1 and 2. Panels C and D illustrate responses to single photophase deletions (cf. Fig. 2 B,C), and panels E and F show responses of mice to skeletons. See text for further details.

proportion (11/13) represents a statistically significant deviation from a model in which the nighttime and daytime components were equally likely to become the new activity onset (Binomial test; $p < 0.05$). The timing of exposure to constant conditions (after the daytime or the nighttime scotophase) had no effect on the pattern of rejoining ($\chi^2 (1) = 0.01$; $p > 0.90$). Controlling for this factor, the asymmetry in rejoining remained statistically significant ($\chi^2 (1) = 6.2$; $p < 0.05$).

Experiment #3: Mice

Of 16 mice, 13 adopted stable split activity patterns in LDLD 7:5:7:5 (Fig. 3A). One mouse (not shown) exhibited a unimodal, conventionally entrained rhythm except during a 2-week period over which activity gradually shifted from the nighttime to the daytime scotophase. Rhythms of the remaining 2 mice were not entrained but instead exhibited relative coordination (Fig. 3B). Among split mice entrained to LDLD 7:5:7:5, the phase angle of entrainment was significantly earlier for the nighttime than for the day-

time activity component (Table 1). When a single evening photophase was replaced with darkness, the distinct bimodal pattern of activity was lost in most of the mice with split rhythms: instead, activity typically persisted for several hours beyond the time of the regular dark:light transition (Fig. 3 C,D). Three days later, a complementary pattern was observed after replacement of the morning photophase with darkness. For both photophase deletions, the split activity pattern was quickly restored, but the component following the deleted photophase sometimes exhibited several transient cycles before it fully resembled the prepulse rhythm.

The longest (3-h) skeleton pulses did not disrupt the overall split pattern of activity (Fig. 3E,F) but in 8 out of 11 cases produced daily activity during 1 of the 2 interpolated 1-h dark pulses (mean duration = 0.70 ± 0.06 h). Activity duration and phase angle of entrainment of the main activity components, however, were unaltered by exposure to these 3-h skeleton pulses (Table 1; $p > 0.10$ for all tests). When skeleton pulse duration was subsequently reduced from 3 h to 2 h,

the stable split activity pattern was disrupted in all 11 mice (Fig. 3E,F). The timing and intensity of activity was markedly altered in one or both of the 5-h scotophases. Absence of a stable rhythm precluded determination of activity duration or phase angle of entrainment.

DISCUSSION

Locomotor activity rhythms of 3 rodent species are shown here to reorganize with activity splitting into 2 separate daily components that may be indefinitely entrained to a 4-phase LDLD cycle. Replacement of individual photophases with darkness did not markedly alter the split rhythm of most Siberian hamsters during probe days, and transfer to darkness several weeks later revealed stability in the split rhythms of all hamsters for a minimum of 2 complete cycles. These results generally mirror those of Syrian hamsters examined in an earlier quantitative study of these responses (Gorman, 2001). More significant, the unchanged activity rhythms in both hamster species exposed to skeleton photoperiods unequivocally demonstrates the entrainment of 2 distinct activity components daily.

For mice, on the other hand, deletion of even a single photophase led to an apparent consolidation of the split rhythm into a single long activity bout. This finding raises the possibility that the apparently split pattern in mice does not actually reflect separate entrainment of 2 subjective nights. Conceivably, mice may have entrained with a long subjective night that spans both scotophases but that is negatively masked by 1 of the 2 intervening photophases. The long subjective night hypothesis is discounted, however, by the finding that individual mice showed the above described behavior after deletions of both the morning and evening photophases instead of after only 1 of the 2. Two Siberian hamsters with the largest perturbations following a photophase deletion were likewise symmetrically affected, but their entrained rhythms were not discernibly different from those of the majority of hamsters. Moreover, these 2 hamsters were unambiguously split under skeleton photoperiods and remained split for several cycles in DD, showing no immediate or short-term perturbations following transfer to DD. Thus, among animals with a split activity pattern, there is interindividual variability in the stability of the pattern, which may also change with time. This continuity in responses between Siberian hamsters and mice suggests that rhythms of the 2 species are similarly split but that the split rhythm in mice is unstable for even half a cycle in darkness.

Although activity is expressed roughly every 12 h, the split entrainment pattern represents the simultaneous expression of 2 differently phased 24-h oscillations rather than a single rhythm with a doubled frequency. The latter hypothesis is contradicted by the slightly different phase angles of entrainment for the 2 activity components (Table 1) and by prior studies of Syrian hamsters demonstrating stable split entrainment to an LDLD cycle where the 2 scotophases were not 12 h apart and where phase angles of entrainment of the 2 components differed more (Evans and Gorman, 2002; Gorman, 2001). The apparent hemicircadian (~12-h) period following splitting in LL likely reflects 2 circadian oscillations in antiphase. In this form of splitting, antiphase oscillations of clock gene expression in the left and right SCN may be the physiological basis, but this remains to be definitively established (de la Iglesia et al., 2000). The physiological basis of the present form of splitting is unlikely to depend on lateral asymmetries in SCN function: Unlike the case of LL-split rhythms, there is no lateral asymmetry of Per1 protein in our LDLD-split hamsters (Meyer-Bernstein, Elliott, and Gorman, unpublished) or of light-induced Fos expression in the SCN in NWR-induced split animals (Gorman et al., 2001). Patterns of SCN Per1 and Per2 expression, moreover, are laterally symmetrical in this latter paradigm (Edelstein et al., 2003).

Each species exhibited small, but statistically reliable, differences in entrainment parameters of the 2 bouts of a split under 1 or more photoperiods. More compelling evidence of functional differences between the 2 oscillators comes from the rejoining behavior of Siberian hamsters in constant conditions, in which the nighttime component nearly always became the unsplit activity onset. An analogous asymmetry has been noted in a similar study of Syrian hamsters following transfer to constant conditions (Elliott and Gorman, unpublished). It remains to be determined whether these differences in oscillator function reflect aftereffects of prior entrainment history or intrinsic properties of the oscillators. Except during probe days, the use of symmetric LDLD cycles ensured that the 2 oscillators would have been exposed to light at very similar phases of their endogenous cycles for more than 10 weeks. We therefore consider it unlikely that aftereffects of the original
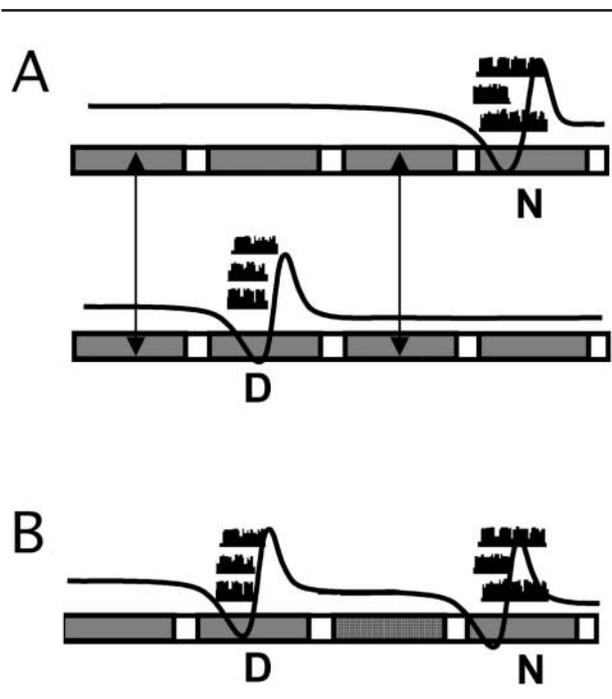
Figure 4. Formal model of the split circadian pacemaker. In (A), the oscillator expressing activity in the nighttime scotophase (N) is depicted above and the comparable oscillator underlying the expression of activity in the daytime (D) scotophase is depicted below. Each oscillator is directly sensitive to light only around the interval of activity that it programs, but the remainder of its cycle is a deadzone with respect to direct phase-shifting of this oscillator by light. The specific shapes of the component PRCs depicted have not yet been determined. Arrows reflect interactions between the 2 oscillators, which may be altered by light exposure. (B) The net output of this system is a split activity rhythm with 2 corresponding and similarly separated phases of response to resetting by light. Thus, the split system may exhibit a system PRC consisting of 2 deadzones separating 2 regions of light response, compressed in duration compared to the normal PRC of an unsplit hamster, but with each displaying delay shifts followed by advance shifts.

entraining conditions would persist through this long interval of entrainment to dictate this functional asymmetry.

As measured by phase shifts and by molecular markers of SCN activity, the circadian pacemaker of the unsplit hamster is highly responsive to light during subjective night but displays a relative deadzone extending throughout most of the subjective day (Elliott and Tamarkin, 1994; Johnson et al., 2003; Kornhauser et al., 1990). The PRC of each of the split oscillators has yet to be characterized in detail, but in a previous study we found that light pulses at the time of each programmed activity bout phase-shift that activity component, whereas light falling during the intervening intervals of inactivity fails to elicit behav-

ioral phase shifts or to induce Fos expression in the SCN (Gorman et al., 2001). If, like that of the unsplit pacemaker, the PRC of each split component exhibits a circadian alternation between light-responsiveness associated with its particular active phase and a deadzone spanning the rest of the circadian cycle, then entrainment of the split pacemaker to LDLD cycles and to skeleton pulses may be straightforward. In this model, each component is phase-shifted daily by the light pulses just before and just after the activity that it programs. As in nonparametric entrainment models, which accurately predict entrainment of the unsplit pacemaker (Pittendrigh and Daan, 1976), the switch from 7-h photophases to 1-h skeleton pulses does not compromise entrainment. The 1-h skeleton pulses that entrain 1 component, however, fall during the deadzone of the 2nd oscillator, which is similarly entrained by light pulses bracketing the 2nd scotophase (Fig. 4).

Whereas the above nonparametric entrainment model would seem to explain well the hamster entrainment patterns, it fails to account for the loss of entrainment with skeleton photoperiods in mice. Approximately one quarter of the mice remained clearly and unambiguously split with 3-h skeleton pulses, while the remaining animals maintained their prior activity patterns with the addition of activity into 1 of the 2 newly intercalated 1-h dark pulses. The transition from 3-h to 2-h skeleton pulses, however, eliminated the split pattern altogether. With the light intensities employed, even 1-h skeleton pulses would be expected to be saturating in terms of classical phase-shifting actions of light, although this argument is based on detailed quantification of light responses in hamsters rather than mice (Nelson and Takahashi, 1999). If these data can be generalized across species, it suggests that entrainment of mice to LDLD cycles likely involves parametric processes whereby light falling during the subjective day contributes to the stability of the split pattern. State variables other than phase of the pacemaker (e.g., amplitude, coupling strength, etc.) may be affected by light, so that the light-pulse PRC of mice need not reflect this putative action (Johnson et al., 2003; Lakin-Thomas, 1995). Although not well characterized, parametric processes have been implicated in entrainment of rodents under nontraditional photocycles (Boulos et al., 2002). Further studies with mice are warranted to assess these possibilities.

Of late, there has been a proliferation of experimental paradigms that illustrate the multioscillatory basis

of circadian rhythms (Abe et al., 1999; Jagota et al., 2000; Mrosovsky and Janik, 1993; Vilaplana et al., 1997). The term *splitting* has been applied to at least 3 circadian preparations (Abe et al., 1999; Boulos and Rusak, 1982; Gorman et al., 2001). Two of these (LL-induced and NWR-induced splitting) are characterized by the temporal resolution of 2 discrete subjective nights as measured in terms of resetting properties of the pacemaker. Because the physiological bases of component oscillators are not yet understood, splitting should be viewed as a descriptive term and not as implying a particular physiological organization, which may differ across paradigms. Similarly, the 2 oscillators entrained by LDLD cycles here bear no known relationship to the evening and morning oscillators identified in photoperiodism research. Any advances in understanding of the physiological bases of component oscillators and the nature of their interactions will contribute importantly to an understanding of the complex pacemaker and of photoperiodism.

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REFERENCES

Abe H, Honma S, Honma K, Suzuki T, and Ebihara S (1999) Functional diversities of two activity components of circadian rhythm in genetical splitting mice (CS strain). J Comp Physiol A 184:243-251.

Aschoff J (1965) Circadian rhythms in man. Science 148:1427-1432.

Boulos Z, Macchi MM, and Terman M (2002) Twilights widen the range of photic entrainment in hamsters. J Biol Rhythms 17:353-363.

Boulos Z and Rusak B (1982) Phase-response curves and the dual-oscillator model of circadian pacemakers. In *Vertebrate Circadian Systems*, J Aschoff, S Daan, and G Groos, eds, pp 203-214. Springer-Verlag, Berlin.

Czeisler CA, Weitzman ED, Moore-Ede MC, Zimmerman JC, and Knauer RS (1980) Human sleep: Its duration and organization depend on its circadian phase. Science 210:1264-1267.

de la Iglesia HO, Meyer J, Carpino A Jr, and Schwartz WJ (2000) Antiphase oscillation of the left and right suprachiasmatic nuclei. Science 290:799-801.

Edelstein K, de la Iglesia HO, and Mrosovsky N (2003) Period gene expression in the suprachiasmatic nucleus of behaviorally decoupled hamsters. Brain Res Mol Brain Res 114:40-45.

Elliott JA and Tamarkin L (1994) Complex circadian regulation of pineal melatonin and wheel-running in Syrian hamsters. J Comp Physiol A 174:469-484.

Evans JA and Gorman MR (2002) Split circadian rhythms of female Syrian hamsters and their offspring. Physiol Behav 76:469-478.

Gorman MR (2001) Exotic photoperiods induce and entrain split circadian activity rhythms in hamsters. J Comp Physiol A 187:793-800.

Gorman MR, Elliott JA, and Evans JA (2003) Plasticity of hamster circadian entrainment patterns depends on light intensity. Chronobiol Int 20:233-248.

Gorman MR and Lee TM (2001) Daily novel wheel running reorganizes and splits hamster circadian activity rhythms. J Biol Rhythms 16:541-551.

Gorman MR, Yellon SM, and Lee TM (2001) Temporal reorganization of the suprachiasmatic nuclei in hamsters with split circadian rhythms. J Biol Rhythms 16:552-563.

Hoffmann K (1971) Splitting of the circadian rhythm as a function of light intensity. In *Biochronometry*, M Menaker, ed, pp 134-146. National Academy of Sciences, Washington, DC.

Illnerova H, Sumova A, Travnikova Z, Jac M, and Jelinkova D (2000) Hormones, subjective night and season of the year. Physiol Res 49:S1-S10.

Jagota A, de la Iglesia HO, and Schwartz WJ (2000) Morning and evening circadian oscillations in the suprachiasmatic nucleus in vitro. Nat Neurosci 3:372-376.

Johnson C, Elliott JA, and Foster R (2003) Entrainment of circadian programs. Chronobiol Int 20:741-773.

Kornhauser JM, Nelson DE, Mayo KE, and Takahashi JS (1990) Photic and circadian regulation of c-fos gene expression in the hamster suprachiasmatic nucleus. Neuron 5:127-134.

Lakin-Thomas PL (1995) A beginner's guide to limit cycles, their uses and abuses. Biol Rhythm Res 26:216-232.

Mrosovsky N and Janik DS (1993) Behavioral decoupling of circadian rhythms. J Biol Rhythms 8:57-65.

Nelson DE and Takahashi JS (1999) Integration and saturation within the circadian photic entrainment pathway of hamsters. Am J Physiol 277:R1351-R1361.

Pickard GE, Kahn R, and Silver R (1984) Splitting of the circadian rhythm of body temperature in the golden hamster. Physiol Behav 32:763-766.

Pittendrigh CS (1988) The photoperiodic phenomena: Seasonal modulation of the "day within." J Biol Rhythms 3:173-188.

Pittendrigh CS and Daan S (1976) A functional analysis of circadian pacemakers in nocturnal rodents: IV. Entrainment: Pacemaker as clock. J Comp Physiol A 106:291-331.

Sulzman FM, Fuller CA, and Moore-Ede MC (1979) Tonic effects of light on the circadian system of the squirrel monkey. J Comp Physiol 129:43-50.

Swann JM and Turek FW (1985) Multiple circadian oscillators regulate the timing of behavioral and endocrine rhythms in female golden hamsters. Science 228:898-900.

Turek FW, Earnest DJ, and Swann J (1982) Splitting of the circadian rhythm of activity in hamsters. In *Vertebrate Circadian Systems*, J Aschoff, S Daan, and G Groos, eds, pp 203-214, Springer-Verlag, Berlin.

Vilaplana J, Cambras T, Campuzano A, and Diez-Noguera A (1997) Simultaneous manifestation of free-running and entrained rhythms in the rat motor activity explained by a multioscillatory system. Chronobiol Int 14:9-18.

Weaver DR (1998) The suprachiasmatic nucleus: A 25-year retrospective. J Biol Rhythms 13:100-112.

Wever RA (1979) *The Circadian System of Man. Results of Experiments under Temporal Isolation*, Springer-Verlag, New York.