

Temporal Reorganization of the Suprachiasmatic Nuclei in Hamsters with Split Circadian Rhythms

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Abstract A dual oscillator basis for mammalian circadian rhythms is suggested by the splitting of activity rhythms into two components in constant light and by the photoperiodic control of pineal melatonin secretion and phase-resetting effects of light. Because splitting and photoperiodism depend on incompatible environmental conditions, however, these literatures have remained distinct. The refinement of a procedure for splitting hamster rhythms in a 24-h light-dark:light-dark cycle has enabled the authors to assess the ability of each of two circadian oscillators to initiate melatonin secretion and to respond to light pulses with behavioral phase shifting and induction of Fos-immunoreactivity in the suprachiasmatic nuclei (SCN). Hamsters exposed to a regimen of afternoon novel wheel running (NWR) split their circadian rhythms into two distinct components, dividing their activity between the latter half of the night and the afternoon dark period previously associated with NWR. Plasma melatonin concentrations were elevated during both activity bouts of split hamsters but were not elevated during the afternoon period in unsplit controls. Light pulses delivered during either the nighttime or afternoon activity bout caused that activity component to phase-delay on subsequent days and induced robust expression of Fos-immunoreactivity in the SCN. Light pulses during intervening periods of locomotor inactivity were ineffective. The authors propose that NWR splits the circadian pacemaker into two distinct oscillatory components separated by approximately 180 degrees, with each expressing a short subjective night.

Key words novel wheel running, melatonin, c-Fos, phase-shift, oscillator interaction

The suprachiasmatic nucleus (SCN) of the hypothalamus is the principal circadian pacemaker in mammals (Weaver, 1998). Although individual cultured SCN cells express circadian rhythms with varying free-running periods and phases (Herzog et al., 1997; Liu et al., 1997; Welsh et al., 1995), in vivo cellular activity from the SCN is largely synchronized (Bouskila and Dudek, 1993). Thus, the question of

oscillator-oscillator interactions would appear to be a central issue in understanding the mammalian pacemaker. A dual-oscillator basis for mammalian circadian rhythms was posited following the observation that prolonged exposure to constant light (LL) induced locomotor activity patterns of hamsters and other species to diverge into two components (Pittendrigh and Daan, 1976). The SCN of split ham-

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sters express comparable split rhythms in multiunit electrical activity, suggesting that splitting is intrinsic to the principal circadian pacemaker (Mason, 1991).

Studies of pineal melatonin synthesis and photoperiodic regulation of activity duration (α) likewise point to a dual oscillator model of circadian rhythms (Elliott and Tamarkin, 1994; Gorman et al., 1997; Illnerova, 1991; Pittendrigh and Daan, 1976). An evening oscillator (E), entrained by evening light, is purported to initiate nocturnal melatonin secretion and locomotor activity, whereas a morning oscillator (M), entrained by morning light, mediates the expression of these functions near the end of night. In longer day lengths of spring and summer, E and M are entrained in a phase relation that generates a short subjective night, while short day lengths of fall and winter allow the phase angle between E and M to increase to generate a long subjective night. Parallel photoperiodic modulation of light-induced phase-resetting of locomotor rhythms, as well as of expression of the immediate early gene *c-Fos* in the SCN, suggests that component oscillators are localized within the SCN (Elliott and Kripke, 1998; Pittendrigh et al., 1984; Sumová et al., 1995), although identification of two distinct oscillators has been lacking. Unfortunately, the clear temporal resolution of distinct oscillators achieved in LL-induced splitting is not seen in the photoperiodism literature, where, with rare exceptions (Jagota et al., 2000), evening and morning oscillator functions may greatly overlap. Conversely, notwithstanding the theoretical milestone it represents, the splitting paradigm has been of limited utility for probing the nature of underlying oscillators largely because its requirement for LL complicates application of the bulk of analytical techniques fruitfully employed in photoperiodism research. Specifically, LL masks locomotor activity, inhibits melatonin secretion, and precludes assessment of acute effects of light pulses on pacemaker function. Thus, these two theoretically important literatures have remained largely separate.

Mrosovsky and Janik (1993) suggested that LL is not the only stimulus capable of splitting rhythms in the hamster: Repeated afternoon exposures to 3 h of novel wheel running (NWR) delayed the onset of nighttime wheel running and, in some cases, led to a transient splitting of locomotor activity rhythms when hamsters were later transferred to constant darkness (DD). Modifying this protocol, Gorman and Lee (2001 [this issue]) recently demonstrated that

activity rhythms could be reliably split and that components of the split rhythms could be entrained to a light-dark:light-dark (LDLD) cycle.

Entrainment of NWR-induced split activity rhythms to an LDLD cycle indirectly suggests that each of the two oscillators mediating the split rhythms can be separately phase-shifted by light. To assess this directly, and to rule out the possibility that extra-SCN oscillators are recruited to mediate one or both activity components (Honma et al., 1989; Stephan et al., 1979), we characterized the effects of light pulses on locomotor activity rhythms and on induction of Fos immunoreactivity in the SCN during each of the two split activity bouts induced by NWR. Additionally, we presented light pulses during the inactive periods between the activity components to exclude the possibility that α was simply lengthened to incorporate both activity components. Finally, models of photoperiodic control of melatonin secretion have posited the existence of distinct evening and morning oscillators timing the initiation and termination, respectively, of the nightly pattern of elevated pineal melatonin secretion (Illnerova, 1991). Because light acutely suppresses melatonin secretion, it has not been possible to evaluate the role of component oscillators split in LL. We therefore assessed whether each of the two split components was capable of initiating melatonin secretion.

MATERIALS AND METHODS

Animals and Husbandry

For all experiments except one, the same 24 male Syrian hamsters (*Mesocricetus auratus*, HsdHan: AURA, Harlan, Indianapolis, IN, USA), 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. These hamsters are the same as used in another separately reported study published in this issue (Gorman and Lee, 2001). Eighteen additional hamsters from the same supplier and housed similarly were used only for the final study of Fos-immunoreactivity in the SCN. Hamsters were housed for 2 to 3 weeks in a 14 h light, 10 h dark condition (LD 14:10; lights on 0500-1900 h) before each regi-

men of daily NWR was used to split activity rhythms. Room illumination at the level of the cage lid varied from 100 to 300 lux.

Split activity rhythms were induced by scheduled exposures to NWR as previously described (Gorman and Lee, 2001). Briefly, after entrainment to LD 14:10, 20 hamsters were transferred daily for 6 to 10 days within the same room to Wahmann wheels 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. With additional days of NWR, onset of nighttime locomotor activity in the home cage is progressively delayed. The number of days of NWR within each experimental run was adjusted so that nighttime activity onset occurred approximately mid-way through the 10-h nighttime scotophase. After the final day of NWR, hamsters were left in their home cages and exposed to DD by leaving lights off after the subsequent 10-h nighttime scotophase. Nonsplitting control hamsters ($n = 4$) housed in the same room experienced identical lighting conditions but were not exposed to NWR and thus never split their activity rhythms.

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. Activity onset (CT 12) was defined as the first bin in each activity bout with wheel revolutions of more than 20, and that was immediately followed by a second interval exceeding this threshold. 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, Seattle, WA, USA) and ClockLab software (Actimetrics, Evanston, IL, USA).

Light Pulse–Induced Phase Shifts of Activity

Changes in the phase response to light following NWR-induced splitting were assessed in three sequential experimental iterations (begun at approximately 12, 18, and 24 weeks of age, respectively). The same cohort of hamsters was repeatedly first entrained to LD 14:10, then exposed to a regimen of NWR to split activity, and then transferred to DD (Aschoff Type II methodology) where they received either a 15-min light pulse or a sham pulse (~450 lux at the level of the cage lid).

Afternoon Light Pulse (CT 13-a)

In the first run, lights remained off after the nighttime scotophase following the final day of NWR (Fig. 1A). Activity rhythms were monitored remotely for onset of wheel running the following afternoon. One hour after onset of this afternoon component of the split activity rhythm, 9 randomly selected hamsters were moved in their home cages to an adjoining room where they experienced the 15-min light pulse. Unpulsed split control animals ($n = 10$) were similarly jostled in their home cages but remained in the same room in darkness. Following the light pulse, animals were returned to the housing room and remained undisturbed for 2 weeks.

Evening Light Pulse between Split Activity Components

Hamsters were next re-entrained to LD 14:10 for 2 weeks and then exposed to an additional regimen of NWR. Following the final day of NWR, lights were turned off 2 h prematurely (1700 EST) to minimize potential phase-shifting effects of the entraining photoperiod and to unmask activity onset in unsplit control hamsters. Of the split hamsters, 10 were randomly selected and pulsed with light for 15 min beginning approximately 1 h after unsplit animals began their normal nighttime activity ($n = 4$). The remaining split hamsters ($n = 9$) served as unpulsed controls: they were similarly jostled at the designated time but not exposed to light.

Nighttime Light Pulse (CT 13-n)

Hamsters were again re-entrained to LD 14:10 for 2 weeks and then induced to split their rhythms with NWR. Following the final day of NWR, hamsters remained in their home cages, and lights remained off after the subsequent nighttime scotophase. Activity was monitored to verify that hamsters exhibited a nighttime activity bout followed by an interval of inactivity and then an afternoon activity bout. Hamsters were pulsed with light for 15 min ($n = 9$) or sham pulsed ($n = 8$) as described above beginning 1 h after the next nighttime activity onset.

Analyses of Phase-Shift Data

Because split circadian rhythms are unstable in DD (i.e., components re-fuse rapidly and τ and phase are

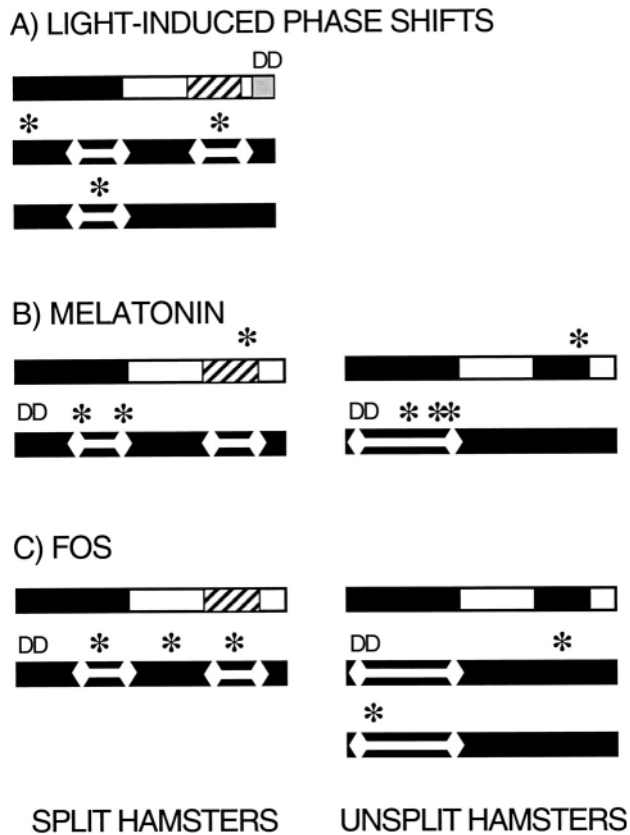


Figure 1. Schematic representation of times of experimental manipulations in hamsters with split activity rhythms and in unsplit controls. Each horizontal bar represents a 24-h period, with dark rectangles reflecting times of lights-off. Hatched rectangles indicate times of novel wheel running (NWR) in darkness. One to 2 days following the final day of NWR are plotted below. Superimposed white dumbbell shapes represent characteristic times of wheel-running activity in DD. Asterisks indicate time of light pulse/sham pulse for determination of behavioral phase-shifting (A), blood sampling (B), or brain collection for SCN Fos-ir (C). In (A), the gray bar indicates that lights were extinguished 2 h early for assessment of the earliest light pulses.

in flux), establishment of a prepulse baseline and calculation of a light-induced phase shift in an individual animal are highly problematic. Therefore, phase shifts were assessed by comparing light-pulsed and sham-pulsed animals in a between-subjects design. For each animal, nighttime and afternoon activity onset of the day preceding the light-pulse or sham-pulse were compared with the corresponding activity onsets on the 2 days following the stimulus (see Fig. 2). Timing of the light pulse was such that we could not obtain a prepulse afternoon activity onset to use as a phase reference in the second experimental run. Hence, phase shifts of this component were not determined. Moreover, activity onsets of the nighttime component were always unambiguous, but as

the experiment progressed, fragmentation of the activity rhythms occasionally precluded identification of a single clear afternoon activity component. In such cases, this component was not analyzed. Last, the free-running period of the nocturnal activity component was computed using least squares regression over the 4 days following the pulse or sham pulse. Because the afternoon component typically rejoined with the nighttime component within this interval, a comparable analysis of the free-running period of the afternoon component was not attempted.

Melatonin in Circulation

To assess whether melatonin secretion patterns were altered by NWR-induced splitting, hamsters were lightly anesthetized with methoxyflurane vapors (Metofane) and retro-orbitally bled in darkness with the aid of a dim red light (< 1 lux). Blood samples were collected into ethylene diamine tetraacetic acid-treated tubes. Plasma was harvested after centrifugation at 5000 rpm for 20 min and was stored at -70°C until assay. Plasma samples were thawed and extracted with dichloromethane, and melatonin concentrations were determined in a single assay as previously described (Bae et al., 1999). The intra-assay coefficient of variation was 14%, and assay sensitivity was 17 pg/ml. Samples below the limit of detectability were assigned values of 17 pg/ml for purposes of graphing and statistics. One to 3 days prior to each of the three runs of NWR described above, blood samples were collected at ZT 16, ZT 20, and ZT 21 from pseudo-randomly selected unsplit hamsters ($n = 3-5/\text{time point}$). After the 6th day of NWR of each experimental run, additional samples were collected at these same times or at ZT 9 from hamsters that were then split ($n = 3-6/\text{time point}$; Fig. 1B). Among unsplit control animals that remained at home, sampling was performed at ZT 9 on the 6th day of the afternoon dark pulse of each run (Fig. 1B). No hamster contributed more than one sample at any given time point, except non-NWR controls at ZT 9. As melatonin concentrations were always undetectable at ZT 9 for these 4 hamsters, only one determination from each hamster was considered in statistical analyses.

SCN Fos-Immunoreactivity

Finally, we assessed whether the temporal pattern of light-induced Fos expression in the SCN was split following exposure to daily NWR. Following the

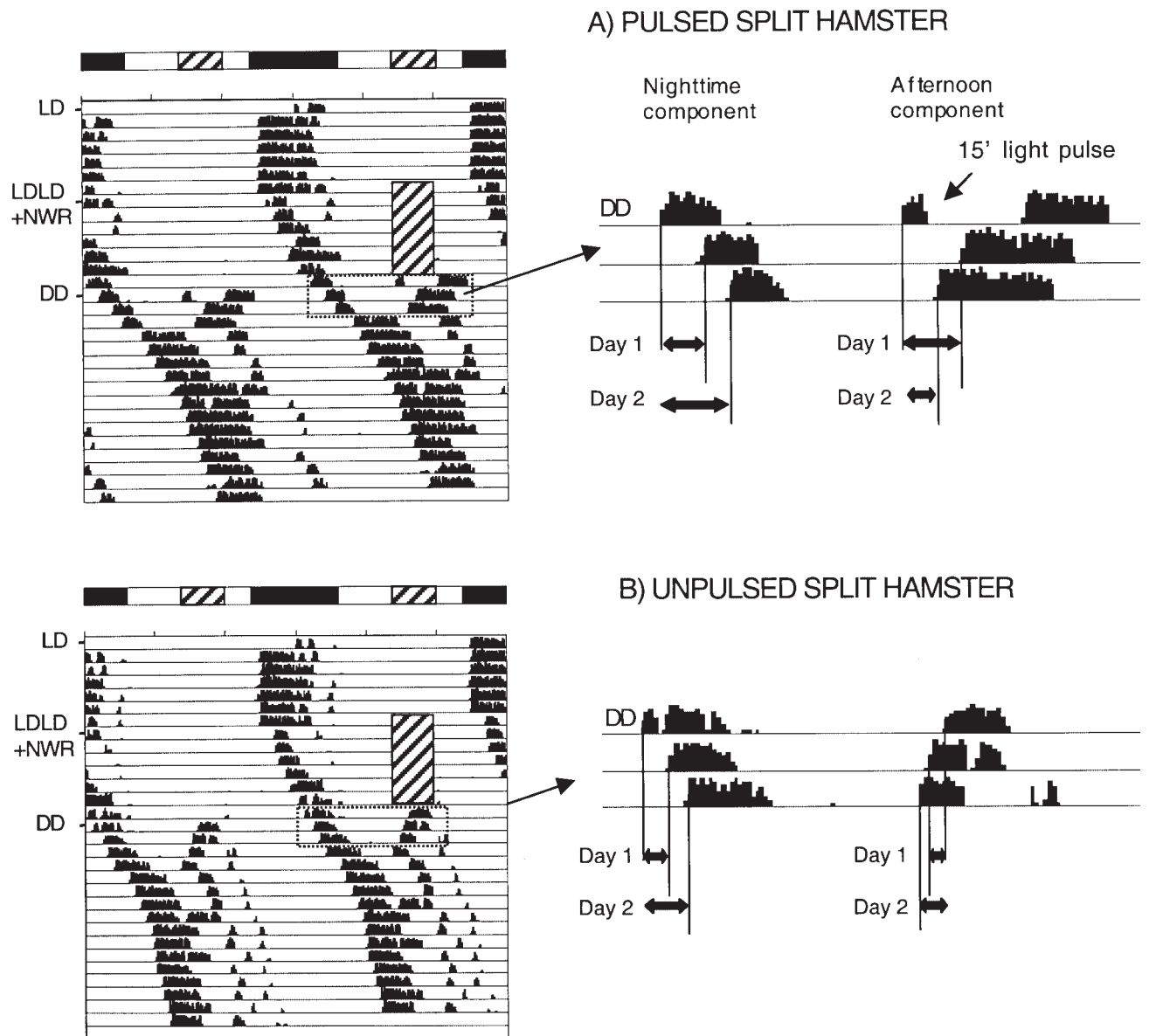


Figure 2. Representative doubled-plotted actograms (left) of hamsters split by novel wheel running (NWR), transferred to DD and pulsed with light (A) or sham-pulsed (B) 1 h after the afternoon activity onset. Data are unclipped and are scaled between zero and maximum revolutions for that hamster. The light-dark cycle prior to transfer to DD is represented above the actogram, with hatched areas representing times of NWR in darkness. Days of NWR are represented by a similar rectangle superimposed upon the right half of the actogram. After NWR, hamsters entered DD beginning during the nighttime scotophase. The first 3 days of DD exposure are enlarged and singly plotted (right) to illustrate method of phase-shift calculation. Double-headed arrows represent Day 1 and Day 2 phase shifts of each component. Onsets of nighttime and afternoon activity on the 1st day in DD served as reference points from which to measure phase shifts. The difference between initial and subsequent activity onsets was measured for each component (net phase shift).

behavioral phase-shifting experiment, hamsters (now 38 weeks of age) were re-entrained to LD 14:10 and exposed to 6 days of NWR. A replicate experiment was subsequently performed with 18 additional hamsters at 12 weeks of age. For both cohorts, hamsters remained at home and lights remained off after the night that followed the 6th day of NWR (Fig. 1C). Hamsters with split activity patterns were randomly

assigned to receive a 15-min light pulse or sham pulse at one of three time points (Fig. 1C; $n = 3-6$ /group): 1 h after the subsequent nighttime activity onset (CT 13-n), during the morning inactive period (break) following nighttime activity (2-4 h after nighttime activity offset), and 1 h after the subsequent afternoon activity onset (CT 13-a). Unsplit control hamsters were also left in DD and were exposed to light or sham-

pulsed the next afternoon at the same time when split hamsters were given their light pulses. Additional unsplit control hamsters received light pulses or sham pulses 1 h after the following nighttime activity onset.

Hamsters were injected with a lethal dose of Nembutal 55 min after the light pulse or sham pulse and perfused intracardially with 40 to 60 ml 0.1M phosphate buffered saline (PBS; pH 7.5) followed by 100 to 150 ml 4% paraformaldehyde in PBS. Brains were postfixed in paraformaldehyde at room temperature for 2 h and transferred to 20% sucrose/PBS. Serial coronal slices were cut at 40 μ m and every fourth section processed for immunocytochemistry. Free-floating sections were incubated successively in 0.1% hydrogen peroxide; 1:1000 anti-fos rabbit IgG (sc-52, Santa Cruz, CA, USA) in PBS with 0.4% Triton-X (Fisher) and 4% normal goat serum for 24 h at 4 °C; 1:200 biotinylated goat anti-rabbit secondary antibody (Vector Labs, Burlingame, CA, USA) for 1 h at room temperature; ABC reagent (Vector) for 1 h; and 0.1% diaminobenzidine (DAB) with 0.02% peroxide for 2 min. Sections were mounted on gelatin-coated slides. The most densely stained section was selected by an observer blind to experimental treatment, and the number of Fos-positive cells in the SCN was counted.

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

RESULTS

NWR and Splitting

In each induction of splitting by NWR, virtually the entire sample (>85%) of hamsters ran robustly in the novel wheels. A subset of the activity data during NWR has been analyzed in detail (Gorman and Lee, 2001) and thus is not reported here. Figure 2 depicts hamsters split by NWR, released into DD, and pulsed with light (A) or sham-pulsed (B).

LIGHT-PULSE INDUCED PHASE SHIFTS OF ACTIVITY

Afternoon Light Pulse (CT 13-a)

The afternoon activity component of pulsed hamsters was phase-delayed relative to that of unpulsed

controls on Day 1 ($p < 0.05$; Figs. 2A, 3A), with a trend in the same direction on Day 2 ($p < 0.10$). The nighttime activity component, which was not itself pulsed, was significantly phase-advanced by this afternoon light pulse ($p < 0.05$ for both days, Fig. 3A). The free-running period of the nighttime component was unaffected by the light pulse (mean $\tau \pm$ SE: 25.08 \pm 0.12 vs. 24.80 \pm 0.18 h for unpulsed and pulsed animals, respectively; $p > 0.20$).

Evening Light Pulse between Split Activity Components

Early evening light pulses (matched to CT 13 of unsplit control hamsters) had no significant effect on the nighttime activity component of split hamsters (Fig. 3B). The free-running period of this component also was not affected by the light pulse (25.11 \pm 0.14 vs. 25.37 \pm 0.20 h for unpulsed and pulsed animals, respectively; $p > 0.30$).

Nighttime Light Pulse (CT 13-n)

On Days 1 and 2, the nighttime activity component of pulsed hamsters was significantly phase-delayed by the 15-min light pulse ($p < 0.005$, $p < 0.01$, respectively, Fig. 3C), compared with sham light pulse controls. On Days 1 and 2, afternoon activity in the split rhythm component was not significantly advanced by light pulses ($p > 0.25$; Fig. 3C). The free-running period of the nighttime activity component was unaffected by the light pulse (mean $\tau \pm$ SE: 24.68 \pm 0.04 vs. 24.55 \pm 0.11 h for unpulsed and pulsed hamsters, respectively; $p > 0.28$).

Melatonin in Circulation

Prior to NWR, plasma melatonin concentrations were detectable in only a minority (4/12) of unsplit hamsters at ZT 16 but had increased above threshold in 9 of 10 animals by ZT 20 (Fig. 4A). One hour later (ZT 21), only 2 out of 12 remained above detectable limits. In contrast, plasma melatonin concentrations of hamsters with split activity patterns in no case (0 out of 10) exceeded the minimum detectable level at ZT 16, a significantly smaller proportion than among controls at this time ($\chi^2 = 4.1$; $p < 0.05$; Fig. 4B). By ZT 21, however, plasma melatonin had increased in 6 out of 10 hamsters, a proportion significantly greater than among hamsters not exposed to NWR ($\chi^2 = 4.4$; $p <$

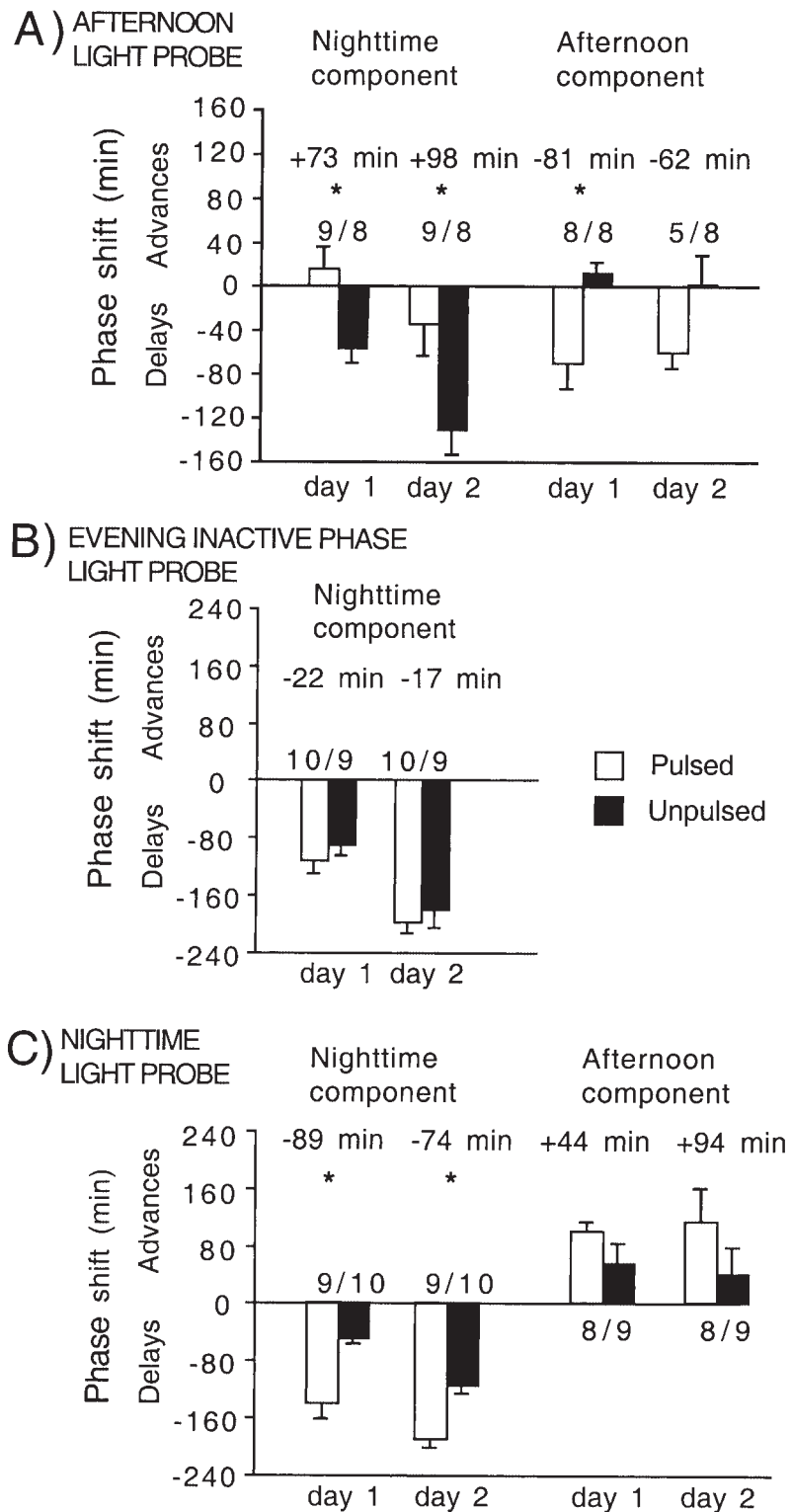
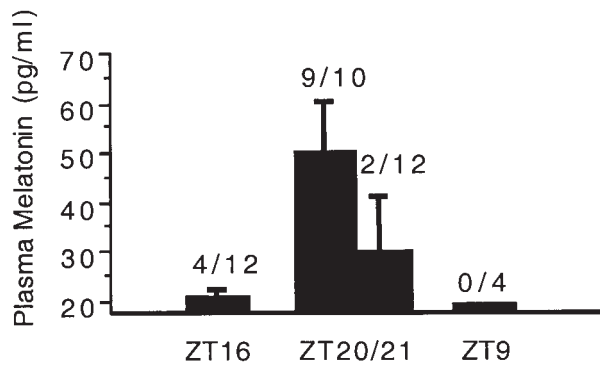


Figure 3. Phase-shifting responses to 15-min light pulses delivered at three phases of the circadian cycle of split hamsters. In each panel, the mean (\pm SE) change in nighttime and afternoon activity onset relative to the first value in DD is depicted for light-pulsed (open bars) and unpulsed (sham) hamsters (filled bars). The net phase shift is indicated above each pair. Sample size is represented below or above bars (pulsed/unpulsed). Asterisks denote significant differences between pulsed and unpulsed animals ($p < 0.05$; Mann-Whitney U ; two-tailed). (A) Day 1 and Day 2 phase shifts after light pulse or sham pulse delivered 1 h after onset of the afternoon activity component (CT 13-a). (B) Phase shifts after pulse or sham pulse during the evening inactive period (see text). Design limitations prevented collection of a prepulse reference value for the afternoon activity component. (C) Phase shifts after pulse or sham pulse delivered 1 h after the nighttime activity onset (CT 13-n).

A) UNSPLIT HAMSTERS



B) SPLIT HAMSTERS

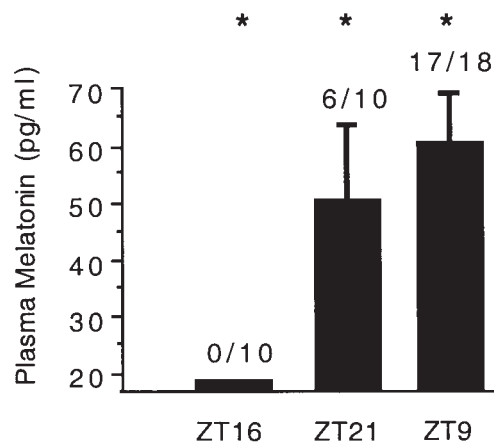


Figure 4. Plasma melatonin concentrations (mean \pm SE) of hamsters with (A) unsplit or (B) split activity rhythms. Sample size and proportion of hamsters with detectable melatonin concentrations are given above histogram. Asterisks denote significant differences ($p < 0.05$) in the proportion of split and unsplit hamsters with elevated melatonin concentrations at that sampling interval.

0.05), but not different from controls at ZT 20. Moreover, after 6 days of NWR, nearly all NWR-split hamsters (17 out of 18) had elevated concentrations of melatonin in circulation at ZT 9. This proportion was significantly greater than in non-NWR controls exposed to similar light conditions where none yielded detectable melatonin titers at ZT 9 ($\chi^2 = 16.6$; $p < 0.001$).

SCN Fos-Immunoreactivity

In hamsters with unsplit activity rhythms, the 15-min light pulse after nighttime activity onset induced significant Fos expression, compared with that in unpulsed controls ($p < 0.05$; Mann-Whitney U ; Figs. 5A, 6A). A light pulse coinciding with the previous afternoon dark period had no stimulatory effect on the number of Fos+ cells in these unsplit hamsters ($p > 0.50$; Figs. 5B, 6A). Hamsters split by NWR, in contrast, showed two periods of increased Fos expression (Figs. 5C, 5E, 6B): Light pulses increased Fos-ir after the nighttime activity onset ($p < 0.05$) and after afternoon activity onset ($p < 0.05$). Moreover, light administered between these two time points had no effect on the number of Fos+ cells ($p > 0.40$; Figs. 5D, 6B). Unpulsed controls at every time point tested—whether split or unsplit—showed minimal Fos expression (Figs. 5F, 6). In groups where Fos was robustly induced by light (split hamsters in the night and the afternoon and unsplit hamsters in the night), there were no differences in the number of Fos+ cells counted ($p > 0.05$). Fos expression was symmetric with respect to the left and right SCN and was concentrated in the ventrolateral SCN in all groups.

DISCUSSION

As in previous experiments, daily exposure to NWR in an LDLD cycle induced split activity rhythms that free-ran and recoupled in DD (Mrosovsky and Janik, 1993). Circadian mechanisms underlying each bout responded similarly to timed light pulses as measured by behavioral phase-shifts or induction of Fos-ir in the SCN, and both activity components were accompanied by elevated plasma melatonin concentrations. The results suggest that NWR temporally reorganizes circadian oscillators within the SCN into two distinct components. We propose that each component oscillator or group of oscillators contains a relatively short subjective night characterized by locomotor activity, elevated melatonin secretion, and light responsiveness.

Conceivably, NWR might have re-entrained the circadian system to generate a long subjective night that spanned the original night and the afternoon scotophase paired with NWR. If one of the two

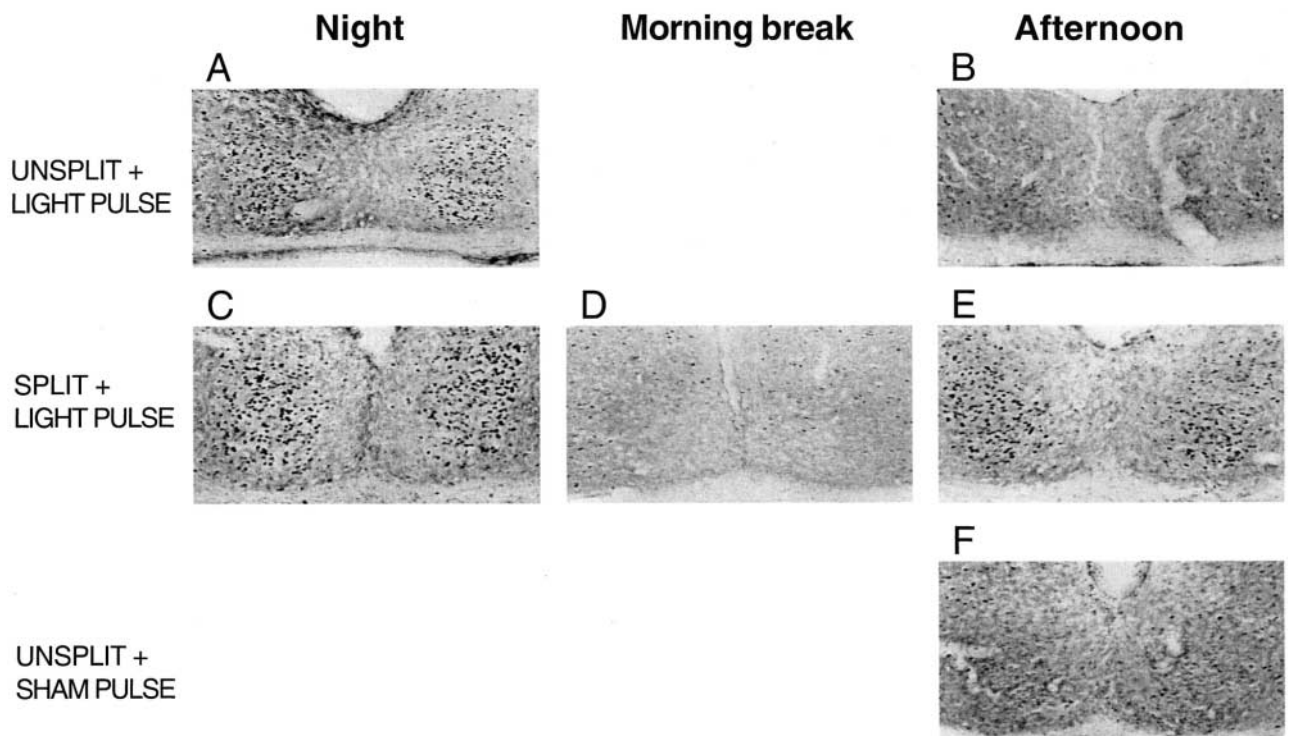


Figure 5. Representative photomicrographs of Fos-immunostained sections from unsplit (A, B, F) and split (C-E) hamsters pulsed or sham-pulsed with light during the nighttime active phase (A, C), the morning inactive period (D), and the afternoon (B, E, F). For the latter time point, split hamsters were active, but unsplit hamsters were inactive.

photophases separating dark periods masked locomotor activity, the rhythm might only appear split. In the present study, light pulses had marked effects when delivered during either activity component but did not induce Fos expression in the SCN during the morning inactive period or shift activity rhythms in the early evening inactive interval. Limited resources precluded assessment of possible light-induced phase shifts during the morning rest period or of SCN Fos-ir expression in the early evening. In unsplit hamsters, however, Fos induction by light is restricted to subjective night and temporally correlates closely with periods of behavioral phase shifting (Kornhauser et al., 1990; Sumová et al., 1995; Travnickova et al., 1996). This close relationship, moreover, is maintained in various photoperiods that alter α . Despite assessment by different methodologies, each inactive period thus appears to represent a dead zone with respect to light responsiveness. These dead zones separate intervals of light responsiveness as measured jointly by behavioral phase-shifting and Fos-ir induction.

Elevated melatonin in circulation during both the subjective afternoon and nighttime bouts of activity in hamsters with split activity rhythms supports the hypothesis that the principal circadian pacemaker is

split by NWR. Among unsplit hamsters, in contrast, plasma melatonin concentrations were elevated in a monophasic pattern. Four hours into the night (ZT 16), 4 out of 12 hamsters had detectable concentrations of melatonin in circulation, consistent with onset of the rising phase in pineal melatonin production in this species (Elliott and Tamarkin, 1994; Goldman et al., 1981). A nighttime elevation in plasma melatonin at ZT 20 was followed by decreases below detectable levels that were manifest by some hamsters at ZT 21 and all hamsters at ZT 9. If NWR delayed the circadian pacemaker as suggested by nighttime activity onsets (Gorman and Lee, 2001) in split hamsters, no elevation of melatonin concentrations at ZT 16 would be expected; importantly, none was found. Increased melatonin secretion clearly occurred later during the nighttime bout of activity. However, the morning decline in circadian melatonin production, evident in the majority of unsplit hamsters at ZT 21, was not observed in split animals, providing further evidence of a delayed nighttime oscillator. Most important, plasma melatonin concentrations were elevated at the end of the afternoon scotophase paired with NWR. Among hamsters exposed to light-dark cycles, darkness in the afternoon to our knowledge has never been

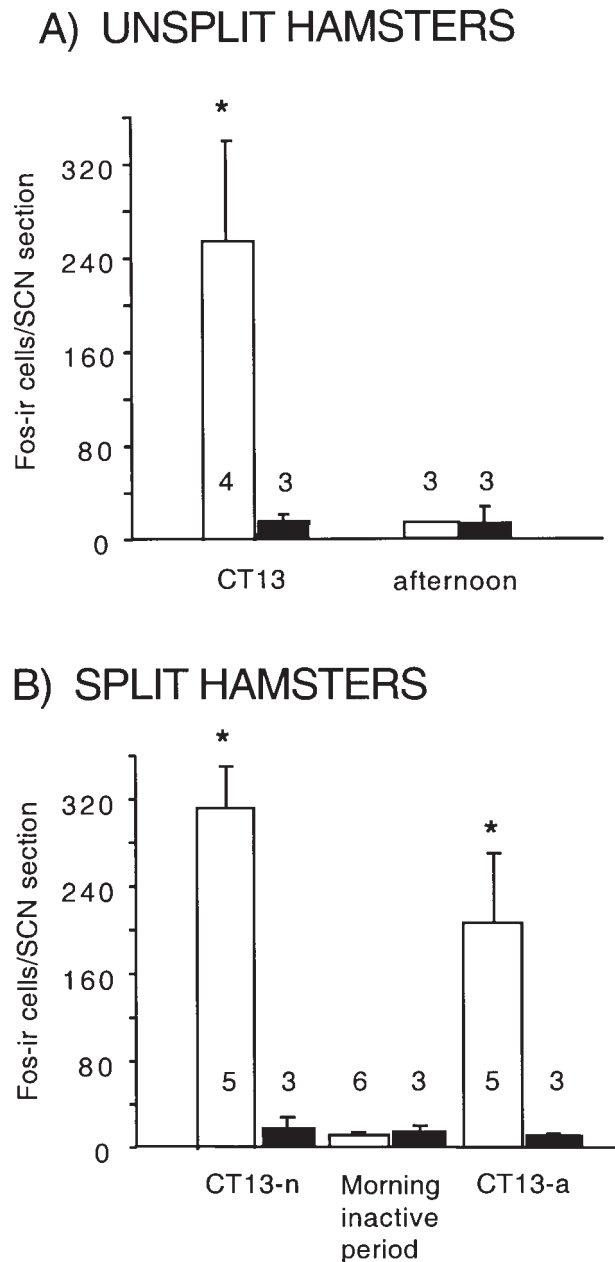


Figure 6. Number of Fos-ir cells/SCN section of (A) unsplit and (B) split hamsters pulsed with light (open bars) or sham-pulsed (filled bars) at various times. Sample size is indicated in or above bars. Asterisks denote significant increases ($p < 0.05$; Mann-Whitney U) in the number of Fos-ir cells relative to unpulsed controls at that time point.

reported to induce melatonin secretion, and no elevation was apparent in nonrunning controls. Thus, these data suggest that the pattern of melatonin, like that of activity, is split by NWR into two components.

The ability of both nighttime and afternoon light pulses to induce Fos expression in the SCN discounts the possibility that split rhythms reflect recruitment of

extra-SCN oscillators that might mediate one of the two activity components. Although their neural substrates are unknown, extra-SCN oscillators are sufficient to generate circadian rhythms in a variety of experimental paradigms (Honma et al., 1989; Stephan et al., 1979). It is not feasible to observe SCN Fos expression in a single hamster during each activity bout, but the uniformity of expression of Fos in the SCN following the afternoon or nighttime light pulse establishes that the principal circadian pacemaker itself responds to light during both activity components. In LL-induced splitting, rhythms of Fos and clock gene expression are out of phase in the left and right SCN (de la Iglesia et al., 2000), although other investigators discerned no left/right differences in electrophysiological activity in the SCN of LL-induced split hamsters (Zlomanczuk et al., 1991). The present Fos data do not suggest an anatomical or physiological distinction between the two oscillators. In the present study, all sections revealed symmetrical induction of Fos in the two SCN, indicating that both left and right are expressing a subjective night simultaneously rather than alternately. Within each SCN, the pattern of Fos induction was also similar between the two bouts, with expression concentrated in the ventrolateral SCN. How each SCN becomes Fos-inducible twice daily is not clear. At the tissue level of organization, the oscillators may be two distinct, but spatially intermixed, cell populations, each responsive to light during either the afternoon or nighttime dark period, but not both. Alternatively, a single population of SCN cells may be induced to express Fos twice daily, perhaps because individual cells in this population contain two oscillators within them, or because they are simultaneously clock-controlled by two other cell populations with rhythms out of phase.

Between-group comparisons demonstrated robust behavioral phase shifts after light pulses. Phase shifts calculated in this fashion might result either from discrete phase shifts or from changes in free-running period (e.g., a lengthening of τ after a light pulse would appear as Day 1 and Day 2 phase-delays by this method). The latter possibility is discounted, however, by the analysis of free-running period in the days following the light pulse. After all pulses, the free-running period was not significantly altered; and in the case of the nighttime pulses, the trend was toward a shortening of τ , which would act counter to the reported phase delays. Thus, phase shifts of the activity components were not secondary to changes in τ but instead reflect discrete phase shifts.

As in LL-induced split animals probed with dark pulses (Boulos and Rusak, 1982; Lees et al., 1983), both NWR-split activity components responded with similar phase shifts to light pulses 1 h after their respective activity onsets. Afternoon light pulses additionally induced phase advances of the unpulsed nighttime component, but pulses in the early nighttime scotophase did not shift rhythms; nor did light pulses in the morning inactive period lead to induction of Fos-ir in the SCN, as is generally a prerequisite for light-induced phase shifts. How is this pattern of responsiveness to be understood? One possibility is that the light-pulse PRC for the nighttime component does indeed contain two light-responsive zones separated by two dead zones. Alternatively, each component may be directly light responsive only during a single short subjective night associated with only one of the two scotophases. The greater fraction of each oscillation may be a dead zone. The observed phase advances of the nighttime activity component following light at CT 13-a, however, could be a secondary consequence of interactions with the afternoon component, which is directly phase-delayed by light. When light phase-delays the afternoon activity component by 90 min as in the current study, the phase angle between the nighttime and afternoon components is altered. If the interaction between the two oscillators depends on their phase relation as commonly presumed, the phase shift of the unpulsed component might reflect this altered interaction with the pulsed component rather than a direct action of light, per se. Future work can develop and test this model of oscillator interactions.

Multioscillatory models of circadian rhythms commonly distinguish between E and M oscillators (Daan and Berde, 1978; Elliott and Tamarkin, 1994; Gorman et al., 1997; Illnerova and Vanacek, 1985; Pittendrigh and Daan, 1976; Sumová et al., 1995). It is not possible to attribute the respective activity components generated here to hypothesized evening and morning oscillators. Regardless of the relation to E and M, however, the circadian timekeeping system can be resolved into two seemingly similar oscillators, both of which can be phase-delayed by light and both of which can drive melatonin secretion within a single 24-h period. Further probing of these two oscillators will complement in vitro work to characterize the suboscillatory basis of the mammalian pacemaker.

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