Phase Angle Difference Alters Coupling Relations of Functionally Distinct Circadian Oscillators Revealed by Rhythm Splitting

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Abstract The interactions (i.e., coupling) between multiple oscillators of a circadian system determine basic properties of the integrated pacemaker. Unfortunately, there are few experimental models to investigate the putative interactions of functionally defined oscillators comprising the mammalian circadian pacemaker. Here the authors induce in hamsters a novel circadian entrainment pattern that is characterized by the daily expression of robust wheel-running activity in each scotophase of a 24-h light:dark:light:dark cycle. The daily activity bouts are mediated by 2 circadian oscillators, here designated "daytime" and "nighttime," that have been temporally dissociated under this light regime. To assess the phase dependence of interactions between oscillatory components, the phase relationship of the 2 daily scotophases was manipulated over a 4-h range, and the timing of activity of the daytime and nighttime components under entrained and probe conditions was examined. The average phase angle of entrainment and the day-to-day variability of activity onset of each activity component depended on the phase relationship of the respective scotophases and not on whether the component occurred in the daytime or the nighttime. Short-term denial of wheel access subsequently influenced amount and duration of wheel running but not timing of its onset, suggesting that only the former measures depend on a homeostatic mechanism sensitive to the time elapsed since prior intense running. Replacement of individual photophases with darkness revealed phase attraction between oscillators that was not dependent on the phase relationship of component oscillators but differed for daytime versus nighttime activity components. Entrainment patterns shown here cannot be accounted for by only nonparametric actions of light. Instead, the phase-dependent interactions of oscillators strongly influence entrainment properties, whereas intrinsic functional differences in dissociated oscillators apparently influence their attraction in darkness. This model system may be ideal for identifying genomic and physiological factors that mediate these interactions and thus contribute importantly to system properties of the mammalian circadian clock.

Key words circadian entrainment, oscillator coupling, hamster, wheel running, splitting, light, activity

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It is hard to imagine how biological systems could guarantee identical intrinsic free-running periods among multiple circadian oscillators (Enright, 1980). Without identical periods, moreover, the relative phases of these units would be continuously changing unless they were somehow synchronized by external zeitgebers or by oscillator interactions. Thus, how multiple circadian oscillators interact with one another is among the most basic questions in chronobiology. The SCN in the anterior hypothalamus of mammals is such a circadian pacemaking system comprising multiple cellular units with diverse free-running periods when isolated in vitro but largely synchronous activity when histotypically integrated (Welsh et al., 1995; Yamaguchi et al., 2003). In the absence of environmental cues, moreover, the SCN is the critical structure that maintains synchrony of a host of organismal rhythms.

Whereas synchronization of its constituent oscillator periods is a first-order requirement for a functional multi-oscillator pacemaker, ecologically motivated studies of rhythm waveform (i.e., shape of the daily oscillation) identify deviation from phase synchrony as an equally important element of circadian pacemakers (Daan and Aschoff, 1975; Pittendrigh and Daan, 1976c). Indeed, seasonal plasticity in circadian rhythmicity has been interpreted as a product of changing phase relations among constituent circadian oscillators. The best-known formulation of a multi-oscillator model is that of Pittendrigh and Daan (1976c), who posited the existence of distinct "evening" and "morning" oscillators.

For 2 or more oscillators to synchronize or to adopt particular phase angle differences, their influence on one another must depend on their phase relationship (i.e., there must be a circadian rhythm to their interaction). If 2 oscillators merely altered each other's period irrespective of their phase relationship, then they would simply free-run with respect to one another, albeit at a different rate than if they were not coupled. Similarly, if coupling permitted each to discretely shift the phase of the other without regard to their phase relationship, they would likewise fail to synchronize. Thus, just as effective entrainment of a pacemaker by a zeitgeber requires phase-dependent resetting, so too does adjustment of phase and period of multiple oscillators via their coupling interactions.

The coupling between well-characterized pacemakers has been studied in various nonmammalian systems (Levine et al., 2002; Page and Nalovic, 1992; Roberts et al., 1987; Steele et al., 2003) and from computational perspectives (Daan and Berde, 1978; Kunz and Achermann, 2003; Oda et al., 2000; Shinbrot and Scarbrough, 1999; Strogatz, 2003; Ueda et al., 2002). A host of basic questions nevertheless remains unanswered: How many oscillators interact? Are component oscillators identical or different? Are their influences reciprocal? Is influence exerted continuously or only at discrete phases? How does light influence this interaction?

Recently, we reported that hamsters and mice under 24-h light:dark:light:dark cycles (LDLD) can entrain with locomotor activity expressed in each of the 2 daily dark phases. This entrainment reflects the temporal dissociation of at least 2 circadian oscillators intrinsic to the SCN (although not the left/right dissociation reported in constant light) (de la Iglesia et al., 2000), with each oscillator programming a short interval of activity in 1 of the 2 dark periods (Edelstein et al., 2003; Gorman, 2001; Gorman and Elliott, 2003, 2004; Gorman et al., 2001). In constant conditions, the 2 activity components rejoin within several cycles apparently under the influence of strong oscillator interactions that can be countered by the 2 daily photophases of the LDLD cycle (Gorman, 2001). The formal basis of this rhythm reorganization has been considered in detail in the cited papers. Because this split paradigm permits identification of the same unambiguous phase marker (e.g., activity onset) for each of 2 oscillations, it represents an attractive empirical model to examine the phase dependence of interactions between component oscillators of the hamster circadian system. We note that these 2 oscillators are not presumed to be equivalent to the evening and morning oscillators described above. On the contrary, each of our behaviorally defined oscillators may itself be a complex oscillator with evening and morning components intrinsic to it (Rosenthal et al., 2005).

Early on in our studies of LDLD splitting, we were struck by the quite similar entrainment patterns of the 2 activity components when the 2 scotophases were arranged in antiphase (i.e., symmetrically positioned 12 h apart) and marked differences in these activity components when the scotophases were asymmetrically arranged (i.e., not 12 h apart; cf. Fig. 1 in Gorman and Elliott, 2003, and Fig. 2 in Gorman, 2001). This observation led us to assess the degree to which entrainment differed as a function of phase angle difference between the 2 oscillator components. Our results reported here demonstrate (1) that classic theory of nonparametric actions by light fails to account for the entrainment patterns of these bimodal rhythms in LDLD, (2) that phase angle difference

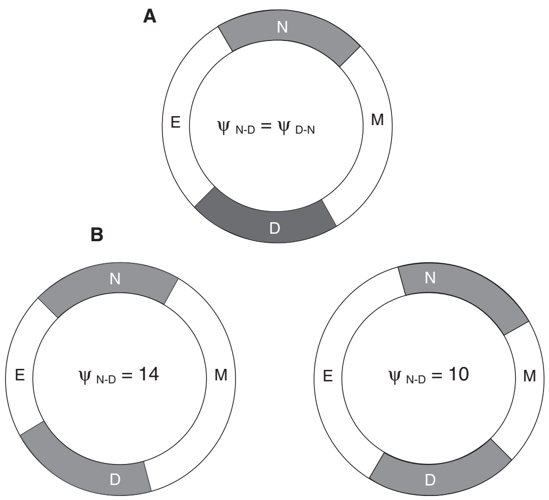

Figure 1. Schematic representation of the major manipulation used in this experiment. Each circle represents a 24-h lighting cycle with periods of light and dark indicated by open and shaded arcs, respectively. In panel A, the Daytime and Nighttime scotophases (dark periods) are 12 h (or 180°) out of phase. Panel B illustrates how these were phase-shifted to create a shorter or longer phase angle difference between the Daytime and Nighttime scotophases. Note that the alternative lighting conditions in panel B can be rotated to produce identical figures but for the labeling of the respective photophases and scotophases. In (B), Daytime and Nighttime are phase leading (i.e., first with respect to the shortest intervening photophase) on left and right diagrams, respectively.

between component oscillators exerts large effects on entrainment to LDLD, and (3) that the 2 underlying oscillators exert asymmetric influence on each other when entraining actions of light are minimized.

MATERIALS AND METHODS

Experimental Protocol

Subjects. Male Syrian hamsters, *Mesocricetus auratus* (*n* = 48; HsdHan: AURA; Harlan, Indianapolis, IN), 4 to 5 weeks of age were group housed in polypropylene cages (27 × 20 × 15 cm) in LD 14:10 (lights-off PST 1900) at 22 °C. Light intensity was 50 to 150 lux during the day and complete darkness (0 lux) at night. Food (Purina Rodent Chow No. 5001, St. Louis, MO) and water ad libitum were available throughout the study.

Induction of splitting. After 2 weeks, hamsters were transferred at 1000 PST (±10 min) to individual cages equipped with 17-cm diameter running wheels located in ventilated, light-tight secondary enclosures holding 12 cages each. The time of transfer coincided with the first scotophase of a new 24-h LDLD cycle (LDLD7:5:7:5) known to induce split activity patterns in hamsters. From this point onwards, a dim green LED (peak wavelength 560 nm; half bandwidth = 23 nm; < 0.1 lux) was illuminated behind every cage throughout all light and "dark" phases, including all experimental manipulations described below. Dim nocturnal illumination comparable in intensity to starlight or dim moonlight markedly increases the splitting response over that observed with completely dark nights (Gorman et al., 2003). Photophase light intensity was 80 to 120 lux.

Beginning after 18 days, at which point all but 1 hamster had adopted a split activity pattern, the phase relationship (i.e., the phase angle difference, Ψ) between the "Daytime" and "Nighttime" scotophases was adjusted for 23 hamsters from $\Psi_{N\text{-}D} = 12$ h to $\Psi_{N\text{-}D} = 10$ h by advancing the Daytime scotophase by 20 min daily for 3 days and simultaneously delaying the Nighttime scotophase by the same amount. For the remaining 24 hamsters, the Daytime and Nighttime scotophases were delayed and advanced, respectively, to yield $\Psi_{N\text{-}D} = 14$. The 2 alternative conditions ($\Psi_{N\text{-}D} = 10$ and $\Psi_{N\text{-}D} = 14$) are identical, except for being phased 12 h apart (Fig. 1). The use of 2 separate nomenclatures facilitates description and analysis of the coupling interactions among component oscillators of LDLD split rhythms. In the first, we designate as "nighttime" and "daytime" the oscillator components mediating activity in the Daytime and Nighttime scotophases, respectively. Throughout, uppercase terminology refers to zeitgeber (i.e., environmental) conditions. Lowercase nomenclature refers to measured activity and presumed endogenous mechanisms. Following changes in $\Psi_{N\text{-}D}$, it is also useful to refer to the "phase-leading" component as the oscillator mediating activity just after the long photophase and to "phase lagging" as the other.

Effects of light manipulations on entrainment. As in past experiments (Gorman, 2001), the entraining effects of the 5-h and 9-h photophases in $\Psi_{N\text{-}D} = 10$ and $\Psi_{N\text{-}D} = 14$ were assessed by replacing either the Evening or Morning photophase with darkness on a single day (see Fig. 2). On day 62, the house lights were not turned on at the usual time after either the Daytime or the Nighttime scotophase, respectively, for animals in each $\Psi_{N\text{-}D}$ (deletion of the Morning photophase in $\Psi_{N\text{-}D} = 14$ is illustrated in Fig. 2C). The lights remained off until the end of the subsequent, normally scheduled scotophase 10 or 14 h later, when the ongoing LDLD cycle was resumed. In an effort to define the critical phase of light action, at day 81,

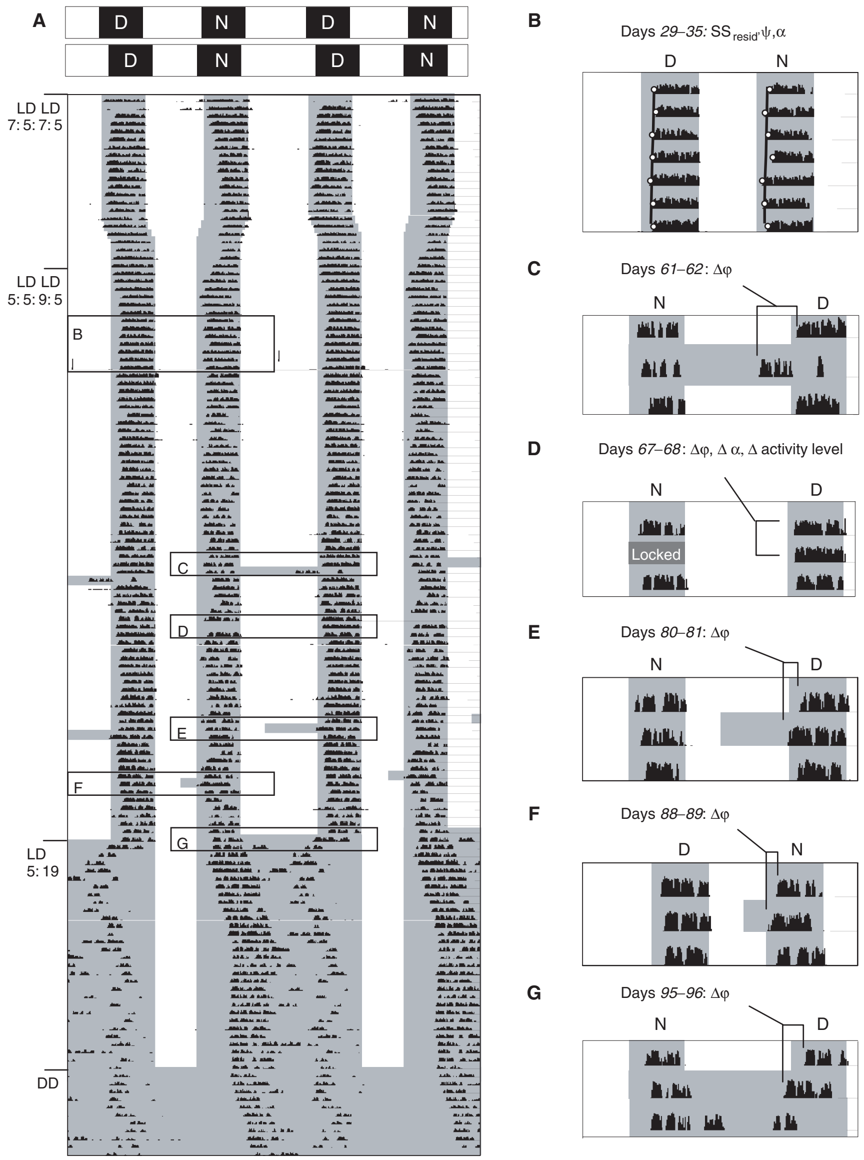

Figure 2. Representative double-plotted wheel-running actogram of a single animal from $\Psi_{N\text{-}D}$ = 14 over the entire course of the experiment (A), as described in the text. The initial LDLD cycle and, below it, the modified LDLD cycle in effect during most of the experiment are indicated by black and white bars above the actogram. Daytime and Nighttime scotophases are abbreviated D and N, respectively. Times of darkness are also indicated by gray shading behind all actograms (A-G). Panels B through G highlight manipulations of lighting and wheel access, as indicated in the text. Measures collected in each of these intervals are noted above the enlarged single-plotted actogram sample. All actograms are unfiltered and scaled from 0 to 150 counts/min.

animals were given abbreviated photophases in lieu of their regular 9-h light phase: animals entrained to each $\Psi_{N\text{-}D}$ thus received either 3L6D (Fig. 2E) or 6L3D on a single day. Approximately 1 week later, each 5-h photophase was replaced on a single day with 2L3D or 3L2D (Fig. 2F). At day 96, 1 of the 2 photophases was replaced permanently with darkness, thereby accomplishing a transfer to 5L19D (Fig. 2G) or to 9L15D, and at day 125, all hamsters were exposed to constant dark. Because groups of 12 hamsters were jointly housed in secondary enclosures with a common lighting condition, complete counterbalancing of all aspects of this study was not achieved. Table 1 identifies the dates of each manipulation for each of the 4 experimental cohorts.

Effects of wheel running on entrainment measures. To assess whether the recency of wheel-running activity influenced the pattern of activity observed in each of the scotophases, wheels of these hamsters were

Table 1. Summary of Lighting Manipulations for Each of 4 Experimental Cohorts

Day	*Manipulation* (n)			
1	$\Psi_{N\text{-}D} = 12$ (47)			
19	$\Psi_{N\text{-}D} = 10$ (23)		$\Psi_{N\text{-}D} = 14$ (24)	
62	Delete 5L (11)[a]	Delete 9L (12)	Delete 9L (12)	Delete 5L (12)
81	6L3D for 9L	3L6D for 9L	3L6D for 9L	6L3D for 9L
89	2L3D for 5L	3L2D for 5L	3L2D for 5L	2L3D for 5L
96	LD9:15	LD5:19	LD5:19	LD9:15
125	DD	DD	DD	DD

a. Replaced with darkness of equivalent duration; sample size is unchanged in subsequent rows.

immobilized throughout a single scotophase by binding rungs of the wheel to the cage lid. On day 67 or 68, wheels were immobilized for every third hamster in each photoperiod for approximately 12 h, beginning 2 to 4 h before the Daytime scotophase. Wheels were immobilized for another third of the hamsters prior to the Nighttime scotophase (Fig. 2D), and wheels of the remaining hamsters were left unmanipulated. Activity onset, activity duration, and total activity were assessed for the scotophases preceding and following wheel immobilization. Animals with unlocked wheels served as experimental controls for both groups. To avoid problems arising from a lack of independence of the control data, effects of Daytime and Nighttime wheel locking were analyzed separately.

Data Collection and Analysis

Each half-wheel revolution triggered a closure of a magnetic contact and was compiled in 6-min bins by VitalView data collection hardware and software (Mini-mitter, Bend, OR). Steady-state entrainment parameters were analyzed over three 7-day intervals, during which there were no changes in experimental manipulations and no cage changes (days 29-35, 71-77, 115-121; see Table 1). To characterize locomotor activity in each of the 2 scotophases (nighttime and daytime activity components), activity onsets were defined as the first bin around the L/D transition with 15 or more counts that was succeeded by 2 additional bins above this threshold. Activity offset was the last of 3 bins around the D/L transition exceeding this threshold. Activity duration (α) was calculated as the difference between offset and onset for each bout on each day. Phase angle of entrainment was expressed as the difference in activity onset relative to the L/D transition (negative values indicate that activity onset follows lights-out).

Cycle-to-cycle variability in activity onsets was quantified over the same 7-day intervals described above. Least squares regression lines were fit through activity onsets, and the sum of squared residuals for each point was calculated. Parallel analysis of statistical variance of the 7 onsets, without regard to their deviation from a linear relationship, yielded identical conclusions, and the results of this analysis are not presented.

Under constant conditions, the free-running period of the activity rhythm—which had fused into an unsplit pattern by this time—was estimated using least squares regression lines through 7 consecutive activity onsets allowing 2 days of transients. Corroborating analyses were conducted using chi-squared periodograms (ClockLab, Actimetrics, Evanston, IL).

Hypothesis Testing

The data were analyzed principally by analysis of variance (ANOVA; Statview 5.0; SAS Institute, Cary, NC). For assessment of steady-state entrainment, the activity onsets of both the nighttime and daytime activity components were treated as repeated measures with the phase angle difference of the 2 scotophases ($\Psi_{N\text{-}D}$) coded as a between-subjects factor. We confirmed that assumptions about heterogeneity of variance were not violated. The same analysis was used for activity duration and total levels of activity of the 2 components.

To assess how each photophase contributes to entrainment, we assessed how the activity onset changed following its replacement with darkness or with an abbreviated photophase, and the 1-day change in activity onset ($\Delta\varphi$; see Fig. 2C-G) was analyzed by 2-factor between-subjects ANOVAs. Factors included the specific activity component analyzed (nighttime vs. daytime) and $\Psi_{N\text{-}D}$.

We tested 3 hypotheses:

Hypothesis 1: Two discrete oscillators are entrained to phase their activity within their respective scotophases exclusively by *nonparametric* actions of light at dawn and dusk. In other words, because each oscillator is exposed to bright light before and after each scotophase, the phase angle difference between them would be of no consequence. This hypothesis would be refuted by a main effect of $\Psi_{N\text{-}D}$ or an interaction of $\Psi_{N\text{-}D}$ and activity component (nighttime vs. daytime). In addition, the presence of a main effect of activity component would indicate that the 2 oscillators are not functionally identical.

Hypothesis 2: The phase angle difference between 2 functionally identical oscillators solely determines their entrainment properties. In other words, only whether an oscillator is phase leading or phase lagging, not whether it is nighttime or daytime, determines how it is entrained to the LDLD cycle. This hypothesis would be refuted by main effects of either activity component or $\Psi_{N\text{-}D}$. Support for this hypothesis would be found in a significant Activity Component × $\Psi_{N\text{-}D}$ interaction. Under this scenario, neither main effect would be significant because the nighttime activity component in $\Psi_{N\text{-}D} = 10$ would be completely balanced by the similarly behaving daytime activity component in $\Psi_{N\text{-}D} = 14$. This relationship is made more obvious by recoding the within-subjects factor in terms of phase-leading (nighttime in $\Psi_{N\text{-}D} = 10$ and daytime in $\Psi_{N\text{-}D} = 14$) and phase-lagging components (daytime in $\Psi_{N\text{-}D} = 10$ and nighttime in $\Psi_{N\text{-}D} = 14$). Under this coding, the aforementioned Activity Component × $\Psi_{N\text{-}D}$ interaction is translated into a main effect of leading versus lagging.

Hypothesis 3: Functionally distinct nighttime and daytime oscillators are *influenced* by phase angle difference, but not exclusively so. In this case, we might expect a significant Activity Component × $\Psi_{N\text{-}D}$ interaction as well as a main effect of 1 or both of the 2 factors.

RESULTS

Splitting Incidence and Entrainment to LDLD7:5:7:5

Within 1 week of transfer from LD14:10 to LDLD7:5:7:5, 47 of 48 hamsters exhibited the split activity pattern described in previous reports. Except for 2 hamsters that expressed a single daily activity bout for less than 1 week just after the transition to $\Psi_{N\text{-}D} = 10$ and $\Psi_{N\text{-}D} = 14$, daily activity was divided between each of the two 5-h scotophases consistently until the LDLD cycle was discontinued near the conclusion of the study. The sole hamster not exhibiting a split rhythm throughout the experiment was not considered in any further analyses.

Entrainment to $\Psi_{N\text{-}D} = 10$ versus $\Psi_{N\text{-}D} = 14$

When calculated from determination of daily values (see Fig. 2B), nearly all entrainment parameters exhibited a strong dependence solely on the phase relationship of the 2 intervals of light and dark. For phase angle of entrainment of each component to its respective scotophase, there was no main effect of activity component or of $\Psi_{N\text{-}D}$ ($p > 0.05$), but these variables strongly interacted, $F(1, 45) = 8.1$ ($p < 0.01$). The leading activity component (i.e., nighttime in $\Psi_{N\text{-}D} = 10$ and daytime in $\Psi_{N\text{-}D} = 14$) exhibited a less negative phase angle of entrainment (i.e., began earlier) than did the lagging component (Fig. 3B).

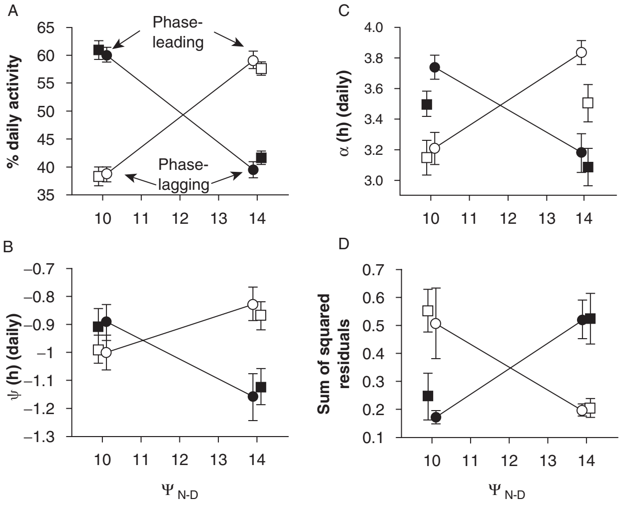

Figure 3. Mean ± SEM entrainment parameters for daytime (open symbols) and nighttime (filled symbols) activity components calculated over days 29 to 35 (circles) and days 71 to 77 (squares). The values have been displaced slightly from their true abscissa value of 10 and 14 h, respectively, to facilitate visualization of values at each time point. Phase-leading and phase-lagging components are labeled in (A). The lines connect daytime entrainment parameters in the 2 $\Psi_{N\text{-}D}$ groups and the nighttime parameters in the 2 $\Psi_{N\text{-}D}$ groups to illustrate the interaction between $\Psi_{N\text{-}D}$ and activity component for days 29 to 35. For statistical results, see text. Phase angle of entrainment (ψ) and activity duration (α) were calculated as the arithmetic mean of activity onsets/offsets determined on individual days (B, C).

Similarly, activity duration did not differ for daytime versus nighttime or under $\Psi_{N\text{-}D} = 10$ versus $\Psi_{N\text{-}D} = 14$, but the interaction was statistically significant with longer activity durations for the leading component, $F(1, 45) = 37.0$ ($p < 0.001$; Fig. 3C). The same pattern held for the percentage of daily activity in each scotophase, $F(1, 45) = 108.0$ ($p < 0.001$; Fig. 3A), and for our measure of day-to-day variation in activity onset (the residual error in the least squares regression line), $F(1, 45) = 18.6$ ($p < 0.001$; Fig. 3D).

When assessed separately several weeks later, each of these statistical results was corroborated. Values from this second interval are plotted without connecting lines in Figure 3A-D. To summarize, as predicted by hypothesis 1, the phase-leading activity component, whether D or N, had a characteristic entrainment pattern that differed from the phase-lagging component.

Replacement of Single Photophases with Darkness

Compared to the onset ~24 h prior, activity onsets occurred significantly earlier in each group when the preceding photophase was replaced with darkness on

Table 2. Mean ± SEM Phase Advance (h) in Daytime or Nighttime Activity Components following Manipulation of the Photophase That Normally Precedes It

	Activity Component	$\Psi_{\text{N-D}} = 10$	*Sample Size*	$\Psi_{\text{N-D}} = 14$	*Sample Size*	
Deletion of entire photophase						
Day 62	nighttime	1.02 ± 0.37	11	1.01 ± 0.26	12	nighttime versus daytime: $p < 0.05$
	daytime	1.16 ± 0.40	12	2.57 ± 0.51	12	
Day 96	nighttime	0.56 ± 0.25	11	0.77 ± 0.40	12	nighttime versus daytime: $p < 0.01$
	daytime	1.63 ± 0.55	12	2.18 ± 0.51	12	
Partial deletion of photophase						
Day 81	nighttime	0.60 ± 0.09	23			nighttime versus daytime: $p < 0.05$
	daytime			0.88 ± 0.09	24	
Day 89	nighttime			1.09 ± 0.20	24	
	daytime	1.35 ± 0.17	23			

day 62 (Fig. 2C; $p < 0.01$; Table 2). The magnitude of this phase advance ($\Delta\varphi$) was significantly greater for the daytime component than the nighttime component (main effect of activity component), $F(1, 43) = 4.7$ ($p < 0.05$). There was no significant effect of $\Psi_{\text{N-D}}$ or the Activity Component × $\Psi_{\text{N-D}}$ interaction. Planned pairwise comparisons revealed that daytime advanced significantly more than nighttime after deletions of the 9-h photophase (diagonal comparison of nighttime under $\Psi_{\text{N-D}} = 10$ vs. daytime under $\Psi_{\text{N-D}} = 14$; $p < 0.05$; Table 2) but not of the 5-h photophase (nighttime under $\Psi_{\text{N-D}} = 14$ vs. daytime under $\Psi_{\text{N-D}} = 10$; Table 2). A directly comparable result was obtained 5 weeks later (day 96) when animals were transferred from LDLD to LD conditions, which, for the first 24 h, is an identical manipulation. These data likewise revealed significant advances in each group ($p < 0.05$), except for the nighttime component in $\Psi_{\text{N-D}} = 10$, for which there was a nonsignificant trend ($p < 0.10$). Significantly greater advances were found for the daytime versus the nighttime component, $F(1, 42) = 8.4$ ($p < 0.01$), but $\Psi_{\text{N-D}}$ had no effect and did not contribute to a significant interaction. Again, planned paired comparisons demonstrated an effect of the activity component following replacement with darkness of the 9-h photophase ($p < 0.05$) but not the 5-h photophase. In summary, as predicted by hypothesis 2, the nighttime and daytime components advanced differentially, and their phase angle difference had no effect.

Replacement of Single Photophases with Abbreviated Photophases

When the 9-h or 5-h light phases were replaced with abbreviated photophases on days 81 and 89 (Fig. 2E,F), each group again exhibited significant phase advances of the activity onset immediately following ($p < 0.01$; Table 2). Replacement of the 9-h photophase with 6L3D versus 3L6D had no differential effect (combined data shown in Table 2). As with full photophase deletions, the onset of the daytime activity component was advanced significantly more than that of nighttime component, $F(1, 43) = 5.2$ ($p < 0.05$; diagonal comparison). When the 5-h photophase was replaced the following week with 2L3D or 3L2D, again there was no effect of 3L2D versus 2L3D (combined data shown in Table 2), and there was no differential effect on daytime versus nighttime components. The results most closely, but do not fully, resemble those predicted by hypothesis 2.

Transfer from LDLD to LD

In the first week of LD9:15 or LD5:19, the split activity rhythms of all animals quickly joined up (Fig. 2G). In the third week of LD, activity onsets of animals in LD5:19 occurred significantly later relative to lights-off than in LD9:15, $F(1, 43) = 234.2$ ($p < 0.001$; Fig. 4A). Prior entrainment history ($\Psi_{\text{N-D}} = 14$ vs. $\Psi_{\text{N-D}} = 10$) had no effect on phase angle of entrainment, nor did this variable interact with photoperiod. For activity duration, there was no main effect of photoperiod or of prior $\Psi_{\text{N-D}}$, but the interaction of these variables was significant ($p < 0.01$) as a result of a long α in LD5:19 among animals from $\Psi_{\text{N-D}} = 10$ (Fig. 4B). Variability of the daily onsets was quite low and unaffected by photoperiod or entrainment history (mean value = 0.169 ± 0.036; cf. Fig. 3F), excluding 1 highly erratic animal with a value that was 6.7 standard deviations from the mean.

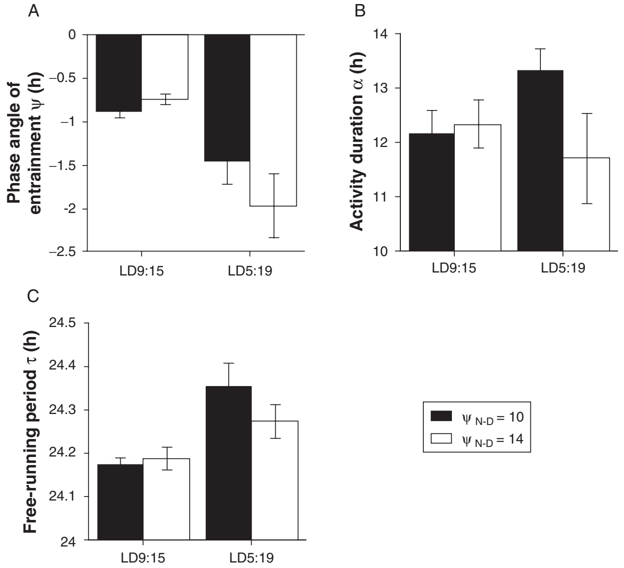

Figure 4. **Mean ± SEM (A) phase angle of entrainment and (B) activity duration in LD9:15 or LD5:19 as a function of prior Ψ_{N-D}. Period of the free-running rhythm (C) under constant conditions is shown in relation to the prior entraining photoperiod and prior Ψ_{N-D}.**

Transfer to Constant Conditions

Under constant conditions, animals previously entrained to LD5:19 had significantly longer free-running periods than those from LD9:15 (Fig. 4C). Whether the activity onset was continuous with the prior daytime or nighttime activity component had no main or interactive effect on period. Excluding 1 animal where ClockLab calculated a dominant periodicity of 38.5 h, the results of chi-squared periodogram analyses provided statistical corroboration of those based on activity onsets.

Wheel-Locking Effects on Subsequent Wheel Running

One cohort of animals with unlocked wheels served as controls both for those animals with wheels locked during the Daytime and those during the Nighttime scotophases. Accordingly, results were analyzed separately for the 2 components so as not to violate assumptions of statistical independence among control subjects. After Daytime wheel locking, wheel revolutions of the nighttime activity component increased 42.8% relative to the cycle before. This increase differed significantly from that in controls, for whom the number of activity counts increased by only 6.5% (Fig. 5A; $p < 0.001$). Relative to the corresponding value on the night prior, activity onset following Daytime blocking was not significantly different from that observed among controls (Fig. 5B). Activity duration, however, was extended by approximately 30 min after wheel locking (Fig. 5C; $p < 0.05$). Blocking wheel running at night produced comparable effects on daytime running parameters, except that the lengthening of activity duration failed to reach statistical significance (Fig. 5A-C). For each measure at each time point, there was no effect of the phase angle difference between components and no interaction of this variable with wheel locking.

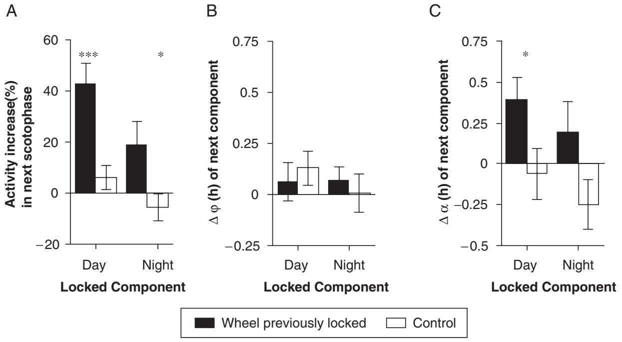

Figure 5. **Mean ± SEM increase in (A) wheel running, (B) phase angle of entrainment, and (C) activity duration in the activity bout that followed the short-term locking of the running wheel. Changes are expressed in relation to values of the same activity component prior to wheel locking. Wheels were never locked for control animals. $^*p < 0.05$. $^{***}p < 0.001$.**

DISCUSSION

This study confirms earlier reports that exposure of Syrian hamsters to 24-h LDLD cycles can induce a stable entrainment pattern in which activity is divided between each of the 2 daily scotophases (Gorman, 2001; Gorman and Elliott, 2003, 2004; Gorman et al., 2003; Gorman and Lee, 2001). The expression of 2 distinct activity bouts under LDLD cannot be attributed to positive or negative masking as the pattern persists under "skeleton photoperiods" (Gorman and Elliott, 2003; Rosenthal et al., 2005) and, as seen in this and earlier studies, after deletions of individual photophases (Gorman, 2001). Extending prior studies, we demonstrate here that the 2 activity components can be readily entrained to 2 LDLD cycles that differ in the phase relationship of their respective scotophases. The entrainment patterns were remarkably stable over the several months of the experiment, despite the fact that groups were repeatedly probed with minor variations in the light environment. Without identifying the underlying neural substrates, the totality of our work in this area establishes that 2 circadian oscillations, each producing a relatively short interval of nocturnal activity, have been temporally dissociated and separately entrained. The formal bases of these rhythms have been discussed in some detail elsewhere (Gorman, 2001; Gorman and Elliott, 2003; Gorman et al., 2003).

Because clock function is inferred indirectly from measurable rhythmic outputs such as wheel running, it is important to distinguish between those aspects of the activity rhythm that closely reflect clock properties and those that are influenced by noncircadian factors. The amount of locomotor activity in any given scotophase is almost certainly not uniquely determined by a circadian mechanism: prevention of running in the prior scotophase significantly increased the total amount of activity and bout duration in the next scotophase. On the other hand, the timed onset of that running was not altered, suggesting that the circadian system gates the onset of activity, but perhaps fatigue or changes in motivation determine the intensity of running and when it ends. We are confident that prevention of running during a single scotophase does not itself induce a substantial change in LDLD-split entrainment at the level of the pacemaker: as monitored by body temperature and general locomotion telemetry, the split activity rhythm is not noticeably compromised by removal of running wheels (Rosenthal et al., 2005). Furthermore, the robustness of activity onset as a reliable marker of clock phase is apparent from its close temporal correlation with onset of light sensitivity and melatonin secretion in rodents with unsplit circadian rhythms (Elliott and Tamarkin, 1994). Activity offset and intensity, on the other hand, are known to be more variable.

The demonstrated control over the phases of the split activity bouts allows critical testing of basic tenets of entrainment theory: specifically, whether separate circadian oscillators are entrained by nonparametric actions of light and whether the phase angle difference between oscillators influences their interactions. Nonparametric resetting of an oscillator by discrete actions of light at dawn and dusk (hypothesis 1) clearly fails to account for major aspects of the present data set. The nighttime activity component, for example, entrains in a markedly different fashion if the 5-h daytime dark pulse begins 14 or 10 h before lights-off (i.e., ZT2 or ZT22, where Nighttime dark onset is defined as ZT12). The same, of course, is true of entrainment parameters for the daytime component. A nonparametric mechanism, contrary to observation, predicts that entrainment parameters would be completely determined by the action of the light pulses (each a minimum of 5 h duration) before and after each scotophase. As there can be no question that the 5- to 9-h photophases are saturating light stimuli in terms of classic phase shifting (Nelson and Takahashi, 1999), the differential entrainment of each component in $\Psi_{N\text{-}D} = 10$ versus $\Psi_{N\text{-}D} = 14$ must be due to parametric actions of light, to phase-dependent interactions with the other circadian oscillator, or to reassortment of component oscillators during reentrainment from symmetrical to asymmetric $\Psi_{N\text{-}D}$ (see below). Arguing against the first possibility is the observation that entrainment in LDLD7:5:7:5 is not much altered by replacement of 7-h photophases with skeleton pulses (Gorman and Elliott, 2003). Violations of nonparametric entrainment theory have likewise been observed in unsplit hamsters exposed to very long, full photoperiods (Pittendrigh and Daan, 1976b).

Behavior of the system in LD and DD further supports the notion that the 2 oscillators interact. If 2 oscillators underlie the split entrainment pattern but they do not interact, then in constant conditions, they would free-run with different periods beating in and out of phase. If they have the same period, of course, they would not rejoin but would free-run in parallel. Empirically, they do neither: following release into constant dark or dim light, 1 split component typically phase delays and the other phase advances so that an interval of inactivity separating components disappears over several days. When the components are no longer distinct, the pacemaker commonly exhibits an altered free-running period (Gorman, 2001; Gorman and Elliott, 2003; Gorman and Lee, 2001; Mrosovsky and Janik, 1993). In the present experiment, transfer from LDLD to LD results in a similar joining of activity components typically via phase delays of the first component in the new longer scotophase and phase advances of the second component, regardless of whether these components are daytime or nighttime or whether they were considered phase leading or phase lagging in LDLD. Moreover, both daytime and nighttime activity components advance when the preceding photophase is deleted or abbreviated. This pattern establishes that any split oscillator is subject to phase-advancing attraction unless light falls in its subjective day. The equivalent effects of 3L6D and 6L3D establish that the critical light effect does not occur in the middle of the regular 9-h photophase. More extensive studies will be required to characterize more fully the phase response of this interaction.

As articulated in hypothesis 2, manipulations of the phase relationship of the 2 scotophases did more than *alter* average and day-to-day variation in activity onset of each component. They reversed them. In other words, the phase-leading and phase-lagging activity components—not nighttime or daytime components—exhibited characteristic entrainment patterns: whether nighttime or daytime, the phase-leading component had earlier and less variable onsets, longer duration and greater total activity. We saw no evidence of gross realignment of split activity components during the transition from symmetric to asymmetric LDLD conditions. Nevertheless, it is possible that oscillators underlying the Ψ_{N-D} = 12 split rhythm reconfigured themselves into different groupings under Ψ_{N-D} = 14 versus Ψ_{N-D} = 10 to produce these characteristic entrainment patterns. In this case, our assumption that each oscillator was effectively phase trapped by the entraining LDLD cycle would be invalid, but the interpretation of the overall results would not be substantively changed: either the entrainment behavior or the assortative phasing of oscillators depends on the phase angle difference of the 2 dark periods.

While no intrinsic difference between the daytime versus nighttime oscillator could be discerned under entrained conditions, one was apparent following light pulse deletions. There, daytime and nighttime oscillators were differentially advanced regardless of their phase angle difference. Thus, consistent with our hypothesis 3, daytime and nighttime cannot be 2 functionally identical oscillators. This differential phase advance is all the more remarkable insofar as it was elicited after entrainment to identical photocycles for weeks or months and thus cannot be reasonably explained as an entrainment aftereffect: with the exception of experimental probe days, the daytime oscillator in Ψ_{N-D} = 14 stood in exactly the same relationship to the light cycle as the nighttime oscillator in Ψ_{N-D} = 10 since day 19 (see Fig. 1B), yet markedly larger phase advances were seen in daytime onsets following light pulse deletions at either day 62 or 96. Also arguing against an explanation based on persistent aftereffects is the minimal influence of prior Ψ_{N-D} on LD entrainment and on free-running behavior under constant conditions. In contrast, a significant aftereffect on free-running period following entrainment to LD9:15 versus LD5:19 was noted. Such photoperiod aftereffects have been reported in other species (Pittendrigh and Daan, 1976a).

The absence of a significant effect of phase angle difference on phase advances after photophase deletion may reflect a lack of statistical power rather than a complete absence of influence. The entrained measures that showed effects of phase angle difference—activity onset and variation—were each derived from 7 measures of onset. Each phase advance, in contrast, was derived from only a single measure and might therefore be a poorer estimate of a true mean value. With respect to phase angle between components, the direction of nonsignificant phase advance differences was highly consistent across replications (Table 2). However, statistical power of these tests reported in Table 2 varied from only 0.05 to 0.57. Thus, much larger sample sizes or larger effect sizes would be required to discern any influence of phase angle difference.

Revisiting the questions raised in the introduction, we conclude that the 2 oscillators mediating activity in LDLD and that may themselves comprise multiple oscillators are not functionally identical (i.e., in terms of periods, PRCs, and/or interactions). Their intrinsic functional differences, however, are counteracted by photophases prior to their programmed activity such that under entraining conditions, their phase relationship determines their entrainment patterns within their respective scotophases. Having demonstrated the utility of this paradigm, in future studies we can map the influence of phase angle difference over a broader range than the 4 h described here. In addition, this technique provides a model with which to study the genomic and physiological factors that may influence this coupling. Elucidation of coupling properties is likely to lead to novel strategies for the manipulation of human circadian rhythms.

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