Behavioral Neuroscience
2010, Vol. 124, No. 1, 87–96

0735-7044/10/$12.00 DOI: 10.1037/a0018088

Dynamic Interactions Between Coupled Oscillators Within the Hamster Circadian Pacemaker

Jennifer A. Evans, Jeffrey A. Elliott, and Michael R. Gorman
University of California, San Diego

Within the mammalian suprachiasmatic nucleus, multiple oscillators interact to coordinate circadian rhythms in behavior and physiology. We have developed a behavioral assay that disassociates central oscillators and allows rigorous study of their formal properties and interactions. Rodents held under 24h light:dark:light:dark (LDLD) cycles display "split" activity rhythms that reflect the reorganization of the central pacemaker into two oscillator groups cycling ~12h apart. After transfer to constant conditions, the two activity components rejoin through a series of transients lasting 2–7 days. Here we analyze fusion dynamics, characterize the underlying oscillator interactions, and assess two influencing factors: phase of transfer and lighting conditions upon transfer. Syrian hamsters were split under LDLD with dimly lit nights and then transferred to constant dim illumination or complete darkness during one of the two daily scotophases. Fusion was influenced by phase of transfer, suggesting that the oscillators split under LDLD exert an asymmetric influence over one another. Transfer to constant dim and dark conditions produced similar overall patterns of fusion, but nevertheless differed in the rejoined state of the system. The present results are discussed within a model wherein oscillators influence one another in a phase-dependent manner.

Keywords: hamster, circadian coupling, split rhythms, dim illumination

The mammalian circadian system is an assembly of oscillators organized by the actions of a neural pacemaker in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Klein, Moore, & Reppert, 1991). The SCN displays robust electrical and biochemical rhythms that persist in individual neurons even after disruption of synaptic communication. Disassociated SCN cells display a broad distribution of circadian periods, indicating that intercellular mechanisms must synchronize oscillators within the network to form a functional pacemaker (Welsh, Logothetis, Meister, & Reppert, 1995; Yamaguchi et al., 2003). Candidates for these synchronizing signals now exist, but a clear understanding of oscillator interactions (i.e., coupling) remains elusive (Aton & Herzog, 2005; Michel & Colwell, 2001), despite its central role in chronobiological research and theory (Kunz & Achermann, 2003; Pittendrigh & Daan, 1976; Strogatz, 2003).

Progress in understanding oscillator coupling in invertebrate species has benefited from the identification of bilateral pacemakers that can be independently monitored and manipulated (Page, 1981; Page & Nalovic, 1992; Tomioka, 1993). In *Aplysia*, the two retinal pacemakers desynchronize after exposure to constant conditions (Hudson & Lickey, 1980), suggesting that they are at most weakly coupled. In contrast, retinal pacemakers in *Bulla* and *Bursatella* appear to have stronger coupling mechanisms because they remain synchronized under constant conditions and reestablish a stable phase relationship after induced phase separations (Roberts, Block, & Lusska, 1987). Studies using a variety of invertebrate models have indicated paired oscillators in these species interact with one another through phase dependent coupling mechanisms (Page, 1981; Page & Nalovic, 1992; Tomioka, 1993; Tomioka, Yamada, Yokoyama, & Yoshihiko, 1991; Wiedenmann, 1983).

In mammals, independent manipulation of multiple pacemakers is not as straightforward as with the peripherally localized clocks of invertebrates. Nonetheless, several environmental manipulations are known to induce temporal reorganization of SCN oscillators controlling behavior. Chronic exposure to constant light (LL) in rodents, for example, can induce a "split" behavioral pattern in which two activity bouts free run in antiphase. LL-induced splitting is associated with antiphase cycling of the left and right lobes of the SCN (de la Iglesia, Meyer, Carpino, & Schwartz, 2000; Ohta, Yamazaki, & McMahon, 2005) as well as intra-SCN reorganization (Yan, Foley, Bobula, Kriegsfeld, & Silver, 2005). Perhaps analogous to bilateral ocular pacemakers in invertebrates, SCN lobes appear to be functionally redundant and are unlikely to differ from one another in terms of their circadian properties (Donaldson & Stephan, 1982; Pickard & Turek, 1983). Maintenance under non-24-hr lighting schedules (e.g., T cycles) can likewise disassociate SCN oscillators in rats. Under these conditions, free-running and entrained behavioral rhythms are mediated by the dorsomedial and ventrolateral SCN, respectively (de la Iglesia, Cambras, Schwartz, & Diez-Noguera, 2004), which

Jennifer A. Evans and Michael R. Gorman, Department of Psychology, University of California, San Diego; Jeffrey A. Elliott, Department of Psychiatry, University of California, San Diego.

Jennifer Evans is now at the Department of Neuroscience, Morehouse School of Medicine. This work was supported by NSF Grant IBN–0346391 and NIH Grant NICHD–36460. We thank Antonio Mora and Robert Sundberg for providing excellent animal care. We are also grateful to our research assistants, Quade French, David Piekarski, and Martin Vakili.

Correspondence concerning this article should be addressed to Jennifer Evans, Department of Neuroscience, Morehouse School of Medicine, 720 Westview Drive, SW, Atlanta, GA 30310. E-mail: jevans@msm.edu

have many known functional and physiological differences (Lee, Billings, & Lehman, 2003; Moore, Speh, & Leak, 2002; Shinohara, Honma, Katsuno, Abe, & Honma, 1995). Last, hamsters and mice will adopt split rhythms under 24-hr light–dark–light–dark (LDLD) cycles, in which each activity bout is entrained to one of the two daily scotophases (Gorman, 2001; Gorman & Elliott, 2003; Gorman & Lee, 2001). The formal properties and dynamics of splitting under LDLD have been described in previous papers (cf., Evans, Elliott, & Gorman, 2005; Gorman, 2001). Instead of left–right antiphase cycling, available data on split rhythms under LDLD suggest the temporal dissociation of oscillators occurs within each SCN lobe (Edelstein, de la Iglesia, & Mrosovsky, 2003; Watanabe et al., 2007; Yan, Silver & Gorman, in press). Relative to other analytical paradigms, LDLD-induced splitting is an attractive model with which to study circadian coupling because splitting occurs in most individuals under permissive conditions (see below), is induced rapidly (<3 weeks), and entrainment of the two bouts is under strong experimental control (Gorman & Steele, 2006).

Among a variety of formal tests (cf. Gorman, 2001; Gorman & Elliott, 2003), the clearest evidence that LDLD dissociates two circadian oscillators derives from the behavior of the system after release into constant conditions, in which the two activity bouts rejoin through a series of transients lasting 2 to 7 days. During the first few days after release into constant conditions, one bout may express a period shorter than 24 hr, whereas the other adopts a period longer than 24 hr. Ultimately the two bouts abut one another and thereafter free run with a common period different from either previously expressed. Because split rhythms can entrain indefinitely under LDLD, one or both photophases of LDLD must counter the oscillator interactions that would otherwise cause the bouts to rejoin. A previous study examining residual interactions between oscillators in LDLD illustrated that the two oscillators exhibit differential, but phase dependent, effects on one another (Gorman & Steele, 2006). Here we examine oscillator interactions unconfounded by bright light by examining fusion of LDLD-induced split rhythms in constant conditions.

Experiment 1 assessed whether rejoining was determined by strong or weak interactions by releasing animals into constant conditions from one of the two daily scotophases. If coupling between oscillators is sufficiently weak that fusion occurs predominantly as a result of different free-running periods of the two oscillators, then the phase of release would be expected to have little effect on the movement of the two respective bouts: one might always advance whereas the other only delays. In contrast, if these oscillators interact strongly on release into constant conditions, the pattern of fusion will likely be influenced by the phase of release. The relative degree of influence of one oscillator over another may then be inferred from the pattern of fusion after release from either phase. We would expect the special case of identical coupling of two identical oscillators to yield two patterns of fusion displaced by 12 hr but otherwise identical.

Experiment 2 assessed whether the dynamic of oscillator fusion differs in dimly lit versus completely dark constant conditions. In tree shrews and crickets, dim light modulates circadian coupling (Meijer, Daan, Overkamp, & Hermann, 1990; Tomioka, 1993). Further, dim illumination equivalent in intensity to dim moonlight markedly alters behavioral rhythms in hamsters under a variety of behavioral assays of circadian coupling (Gorman, Evans, & Elliott, 2006). For example, nearly 100% of Syrian hamsters will split under LDLD with dimly lit nights, but only 25 to 33% do so when housed with completely dark nights (Evans et al., 2005; Gorman, Elliott, & Evans, 2003). Because the induction of split rhythms is strongly modulated by dim light, we assessed whether rejoining would be affected by whether constant conditions were dark or dimly lit.

Method

Subjects

Syrian hamsters (*Mesocricetus auratus*) were either obtained from Harlan (Experiment 1, HsdHan: AURA, Indianapolis, IN) or bred from stock originally purchased from this vendor (Experiment 2). Prior to use in experiments, animals were group housed inside polypropylene cages (48 × 27 × 20 cm) without running wheels and entrained to a 14:10-hr light–dark cycle (Experiment 1: lights on 0700 PST, Experiment 2: lights on: 0300 PST; photophase light intensity: 100–300 lux, scotophase light intensity: 0 lux). Ambient temperature was maintained at 22 ± 2 °C, with food (Purina Rodent Chow 5001, St Louis, MO) and water available ad libitum. Experiments were conducted in compliance with all the rules and regulations of the Institutional Animal Care and Use Committee, University of California, San Diego.

LDLD-Induced Split Rhythm Generation

To induce splitting, hamsters were transferred to LDLD at the start of the new "daytime" scotophase (DS). The term *scotophase* is used because the daily "dark" periods were in fact dimly lit under LDLD to induce splitting. Ventilated, light-tight chambers held one (Experiment 1) or 12 (Experiment 2) individual cages (Experiment 1: 27 × 20 × 15 cm; Experiment 2: 48 × 27 × 20 cm) containing a running wheel (17 cm diameter). One week after transfer to LDLD, cages were exchanged during the first 90 min of the DS, which often triggers splitting in animals that had not split prior to the cage change (Gorman et al., 2003). Each animal was relocated to a new cage with fresh food and water under the direction of a dim-red headlamp (<2 min exposure/animal). Cages were changed at this phase using an identical procedure once every 2 to 3 weeks thereafter until release into constant conditions.

Dim-Lighting Conditions

Dim illumination was provided to all animals by green narrow-band light-emitting diodes (LEDs, 0.03 W, 12V, Product LH1049–3702, Arcolectric, Thousand Palms, CA) mounted externally and facing the back wall of each cage. These LEDs emit a peak wavelength of 560 nm, with a half maximum bandwidth of 23 nm (Ocean Optics PS1000 spectrometer; Dunedin, FL). Dim illumination was measured with the photometer sensor (IL1700 Radiometer system, International Light, Newburyport, MA) placed within the running wheel and oriented toward the LED to estimate dim light levels experienced by hamsters while active in the brightest area of the home cage. For Experiment 1, average luminance was 4.2×10^{-3} lux, which is equivalent to an irradiance of 6.22×10^{-10} W/cm^2 and a photon flux of 1.8×10^9 photons/cm^{2}sec^1. In Experiment 2, average luminance was 9.0×10^{-3} lux,

which is equivalent to an irradiance of 1.32×10^{-9} W/cm^2 and a photon flux of 3.7×10^9 photons/cm^{2}sec^1.

Experiment 1—Phase of Release

Male hamsters ($N = 40$, 26–27 weeks old) were induced to split under LDLD 7:5:7:5 (lights off: 1400, 0200 PST). Eight weeks later, a subset of split animals ($n = 16$) were selected for this experiment and randomly assigned to be transferred to constant dim light at either the beginning of the DS ($n = 8$) or nighttime scotophase (NS, $n = 8$). Animals remained undisturbed in constant conditions for 2 weeks.

Experiment 2—Scotopic Condition

Male hamsters ($N = 24$, 10–11 weeks old) were induced to split under LDLD 8:4:8:4 (lights off: 1030, 2230 PST), which our experience suggested would produce more stable split entrainment than LDLD 7:5:7:5. Five weeks after transfer to LDLD, split hamsters were released into either constant dim illumination (DIM, $n = 11$) or darkness (DARK, $n = 11$) at the start of the DS. Animals remained undisturbed in constant conditions for 2 weeks. Four months later, a separate group of male hamsters ($N = 22$) was likewise transferred to LDLD 8:4:8:4 at 10 to 11 weeks of age, then released at the beginning of the NS into DIM ($n = 11$) or DARK ($n = 9$) for 3 weeks.

Data Collection

Half revolutions of home cage wheels triggered closures of a magnetic reed switch, which were recorded and compiled into 6-min bins by Dataquest III or VitalView software (Mini-Mitter, Sun River, OR). Actograms were prepared and analyzed using ClockLab software (Actimetrics, Evanston, IL).

Analyses of Split Entrainment

As in past studies, splitting was defined as expression of wheel-running bouts > 30 min entrained to each scotophase for at least 5 consecutive days. There was no ambiguity in classifying animals as split, and consistent with previous research, nearly all animals in each of the following experiments exhibited split rhythms shortly after transfer to LDLD. Split entrainment during the last week of LDLD was quantified using bout analyses to define the asymmetry of the split activity pattern (see Figure 1). Starting 3 hr before lights off for each scotophase, activity onset for that activity bout was identified as the first 6-min bin above a threshold value of 15 counts/min followed by two consecutive bins above threshold. Activity offset was determined by a similar but opposite rule. For each split activity component, the bout length (BL) was taken as the temporal difference between activity offset and onset (NS_{BL}, DS_{BL}). Phase angle of entrainment for each bout was quantified as the temporal difference between the closest light-to-dark transition and activity onset of that bout ($NS\psi_{L/D,}$ $DS\psi_{L/D}$). To assess whether activity under LDLD was symmetrically distributed between split activity components, the ratio of the DS and NS bout length (DS_{BL}/NS_{BL}) was calculated. In addition, the number of hours between the average onset of the NS and that for the DS (ψ_{NS-DS}) was determined.

Analyses of Fusion

Because it can be difficult to identify visually the exact cycle on which fusion is complete, the latency to the fused state may be

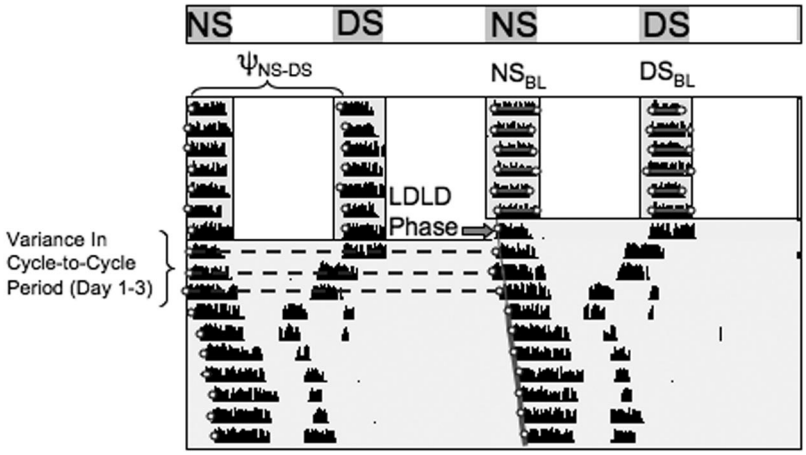

Figure 1. Schematic illustrating measures of split rhythm entrainment and fusion after release into constant conditions. The average bout length (BL) for each split activity component (NS_{BL}, DS_{BL}) was determined by identifying activity onset and offset for each activity bout during the last week under 24-hr light–dark–light–dark cycle (LDLD). The average phase difference between the NS and DS activity bouts was also determined (ψ_{NS-DS}). After release into constant conditions, cycle-to-cycle period was determined using the difference in time between two consecutive onsets (dashed lines) and variance was derived using a 3-day moving window (as illustrated for Days 1 to 3). Last, the phase of the LDLD cycle from which the fused rhythm derived (LDLD phase, arrow) was determined from a linear regression fit to five to seven consecutive activity onsets (thick line, excluding the first four days after release) extrapolated backward to the first day after release from LDLD (thin line). For this particular animal, the split rhythm appears to be fused within 6 days after release into constant conditions. NS = nighttime scotophase; DS = daytime scotophase.

operationalized by the number of cycles until circadian period and waveform stabilize. Whereas the fused rhythm has one unambiguous activity onset and one unambiguous activity offset, this is not the case in the first few cycles after release into constant conditions. Thus, for purposes of characterizing the rejoining transients, we identified these parameters for the fully joined system on the last full cycle under constant conditions and worked backward (i.e., earlier) using the threshold criteria described above and the principle of continuity (see Figure 1). Cycle-to-cycle measures of circadian period and waveform were calculated for the 2 weeks following release into constant conditions. For each cycle, the length of the active phase (α) was calculated from the difference between activity offset and onset. Period was calculated on a cycle-to-cycle basis from the number of hours separating two consecutive activity onsets. Variance in cycle-to-cycle period was calculated with a 3-day moving window. We also determined the phase of the LDLD cycle from which the fused rhythm derived (see Figure 1). Period of the fused rhythm was measured by the slope of a regression line fit to 5 to 7 consecutive activity onsets, excluding the first 4 days after release into constant conditions. Using the phase and slope of this regression line, activity onset was projected backward to the last day under LDLD, and the retrojected phase of activity onset was recorded for each individual animal. The LDLD phase of retrojected activity onset is plotted in angular degrees, with 0° being the midpoint of the NS.

Statistical Analyses

Most statistical tests were conducted with JMP software (SAS Institute, Cary, NC). For each experiment, split entrainment measures (DS_{BL}/NS_{BL}, ψ_{NS-DS}) were analyzed with a one sample t test to determine whether the average BL ratio was different from $\mu = 1$ and whether the average ψ_{NS-DS} was different from antiphase (i.e., $\mu =$ 12 hr). Split entrainment measures (i.e., $NS\psi_{L/D}$, $DS\psi_{L/D}$ DS_{BL}/NS_{BL}, ψ_{NS-DS}) were correlated with measures of fusion to determine if the fusion of split rhythms is related to individual differences in entrainment prior to release. Continuously varying circadian measures recorded under free-running conditions (cycle-to-cycle α and variance in cycle-to-cycle period) were initially assessed using repeated-measures analysis of variance (ANOVA), then post hoc tests were conducted using full-factorial ANOVA with Least Squared means contrasts using the Bonferroni correction to control for multiple comparisons. In addition, the Raleigh test was used to test for randomness of the distribution of activity onsets projected back to the last day under LDLD (Batschelet, 1981).

Results

Experiment 1—Fusion After Release From the Nighttime or Daytime Scotophase

In Experiment 1, split rhythms were characterized by two statistically symmetric activity bouts ~12-hr apart (Table 1, hypotheses as described in Method, $p > .05$). Release into constant dim light from the NS or DS resulted in two different patterns of fusion (see Figure 2). In general, it was easier to distinguish two distinct activity bouts on cycles immediately after DS release than after NS release. Moreover, relative to NS release, the pattern of fusion after DS release was more variable between animals. In all animals released from the NS, activity onset of the fused rhythm was continuous with the NS and activity offset was continuous with the DS. The only salient variation among animals released from the NS is whether activity offset advanced gradually or suddenly (cf. Figure 2A and 2B). In contrast, for the majority of animals released from the DS (5/8 animals, Figure 2C), activity onset of the fused rhythm was continuous with the activity bout entrained to the DS. In many of these records, a large activity bout appeared near the phase of the DS and advanced over the first 2 to 3 cycles. Concurrently, a smaller activity bout appeared near the former phase of the NS and delayed over the next 2 to 3 cycles before disappearing. In the remaining animals released from the DS, activity onset of the fused activity rhythm was continuous with the NS (Figure 2D).

Table 1
Split Entrainment Measures in Experiments 1 and 2

Experiment	DS_{BL}/NS_{BL}	ψ_{NS-DS}
1	1.09 ± 0.1	11.76 ± 0.2 hr
2	1.24 ± 0.1*	11.44 ± 0.1 hr**

Note. DS = daytime scotophase; BL = bout length; NS = nighttime scotophase; ψ_{NS-DS} = average phase difference between NS and DS. Differs significantly from 1.0* or 12 hr**, $p < .05$.

Consistent with qualitative differences in the general pattern of split fusion after NS and DS release, quantitative differences were observed. For nearly every animal released from the NS, activity onset of the fused rhythm projected back to a phase within the NS, whereas the retrojected phase of activity onset after DS release was more variable (Figure 3A, Levene test, $p < .01$). Consequently, the retrojected phase on the last day of LDLD was significantly clustered for NS release, but not for DS release (Raleigh test, $p < .001$ and $p > .1$, respectively). The average phase of retrojected onsets for DS release was ~8 hr after the NS, which was significantly different from the activity onset after NS release, (χ^2 test, $p < .05$) and significantly different from a phase 12 hr after the average activity onset after NS release, (χ^2 test, $p < .001$). Thus, the average activity onsets after DS and NS release were neither in phase nor in antiphase (differing by neither 0 hr or 12 hr). Period of the fused rhythm used to assess retrojected phase of activity onset was not affected by the phase of release (see Table 2).

By tracking changes in period and waveform during split fusion, we find evidence that animals released after the NS stabilized faster than animals released after the DS. Variation in cycle-to-cycle period reached low levels shortly after NS release, but this measure remained high and more variable for more cycles after DS release (Figure 3B, repeated-measures ANOVA, Release*Time: $p < .05$). Over the first few days after release from both the DS and NS, the duration of the active phase (α) decreased as split bouts consolidated, which coincided with increases in the duration of the inactive phase (see Figure 2). However, on the first 3 days after release, α was longer after DS release than after NS release (Figure 3C, repeated-measures ANOVA, Release*Time: $p < .05$).

Experiment 2—Fusion After Release Into Constant-Dim or Dark Conditions

In Experiment 2, split activity bouts were not symmetric in length or phase angle (Table 1, $p < .001$). Consistent with Exper-

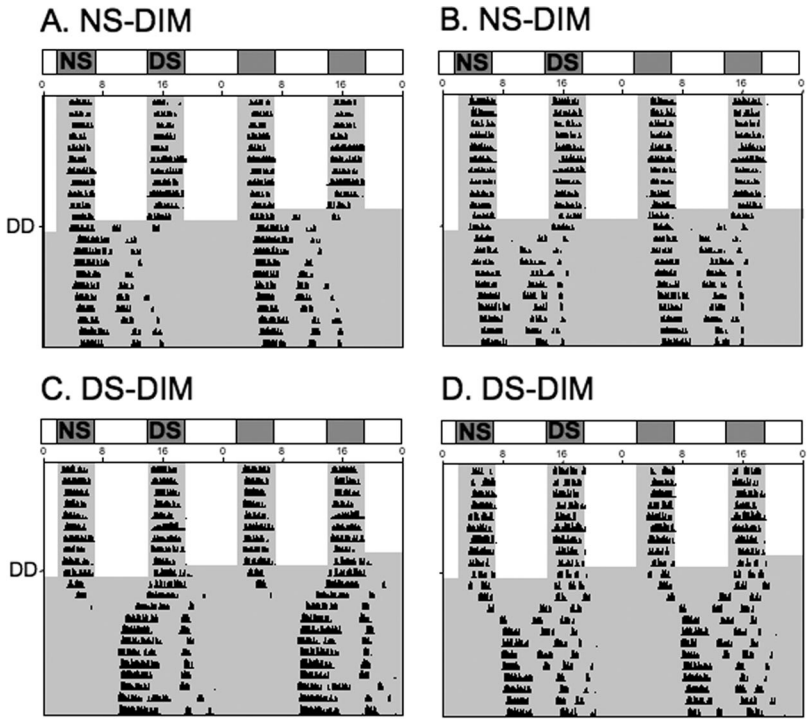

Figure 2. Representative wheel-running actograms illustrating fusion of 24-hr light–dark–light–dark cycle (LDLD)-induced split rhythms after release from the nighttime scotophase (NS) or daytime scotophase (DS) into constant dim illumination in Experiment 1. Light–dark bars at the top of each actogram, along with internal shading, represent the lighting conditions in place during the experiment.

iment 1, different patterns were again observed after NS and DS release and overall, animals released into dim and completely dark conditions exhibited similar patterns of fusion (Figure 4A). Once again, for every animal released from the NS, activity onset of the fused rhythm was continuous with the NS. For the majority of animals released from the DS, activity onset of the fused rhythm was continuous with the DS (9/11 animals for both DIM and DARK groups).

Release into constant DARK or DIM did not influence the average LDLD phase or the within-group variation after either release phase (Figure 4B). Although not run concurrently, after DS release there was again more group variability than after NS

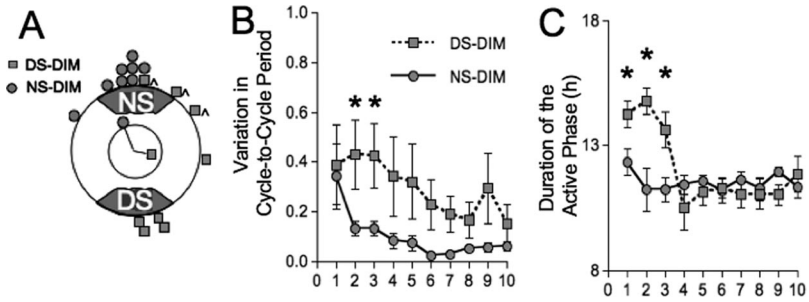

Figure 3. Quantitative measures of fusion of 24-hr light–dark–light–dark cycle (LDLD)-induced split rhythms after NS or DS release into constant dim illumination in Experiment 1. (A) Phase distribution of activity onsets, plotted in angular degrees, on the last day under LDLD, as determined using the free-running period calculated on Days 4 to 9 postrelease. Lines represent mean angular vectors and those extending outside the inner circle indicate significant clustering for that group as determined by the Raleigh test. ˆ indicates DS animals that displayed a fused rhythm continuous with the NS. (B) Variation in cycle-to-cycle period after release, as measured by a 3-day moving window (number of days after release represents first day in this 3-day moving window). (C) Cycle-to-cycle duration of the active phase after release. * Least Squared means contrasts, $p < .05$. NS = nighttime scotophase; DS = daytime scotophase.

Table 2
Period (hr ± SEM) of the Fused Rhythm

Experiment 1	DS	NS	*p* value
	24.09 ± 0.04	24.12 ± 0.04	>.5
Experiment 2	**DIM**	**DARK**	***p* value**
DS	24.16 ± 0.04	23.99 ± 0.03	<.05
NS	24.14 ± 0.02	24.01 ± 0.03	<.05

Note. DS = daytime scotophase; NS = nighttime scotophase; DIM = constant dim illumination; DARK = darkness.

release (Figure 4B, Levene test, $p < .01$, for both DIM and DARK groups) and average activity onset after NS and DS release was not in phase or in antiphase (χ^2 test, $p < .001$, for both DIM and DARK groups). Unlike Experiment 1, activity onsets on the last day of LDLD were significantly clustered for both NS and DS groups released into either DIM and DARK conditions (Figure 4B, Raleigh test, $p < .001$ for each of the four groups).

In addition, changes in the variation of cycle-to-cycle period did not differ between animals released into DIM and DARK conditions from either phase (Figure 5A, NS: repeated-measures ANOVA, $p > .2$; DS: repeated-measures ANOVA, $p > .7$). Like Experiment 1, variation in cycle-to-cycle period reached lower levels sooner after NS release than after DS release (repeated-measures ANOVA, Release*Time: $p < .01$, for both DIM and DARK groups) but all groups displayed low and stable levels within a week of release. Dim illumination lengthened the period of the fused rhythm after both NS and DS release (Table 2; full-factorial ANOVA, SC: $p < .05$, release, SC*Release: $p > .25$).

Cycle-to-cycle changes in circadian waveform were affected by lighting conditions on release in a manner that depended on the phase of release (Figure 5B). After NS release, both DIM and DARK groups decreased α to ~10 hr, but NS–DARK animals showed shorter α than DIM cohorts on the first few days after release (repeated-measures ANOVA, $p < .05$). Like after NS release, DS–DIM animals adopted an α of ~10 hr by the third day, whereas DS–DARK animals showed a significantly shorter active phase (α of ~8 hr) on the third, fourth, and sixth day after release (repeated-measures ANOVA, $p < .005$).

Last, we assessed whether measures of fusion were related to asymmetries in split entrainment. Split entrainment measures were correlated with fusion after NS release, but not DS release, despite the observation that the latter was associated with more within-group variation (cf. Figure 4B). After NS release, retrojected LDLD phase occurred later within the NS animals when the DS was the larger of the two split bouts (i.e., $DS_{BL}/NS_{BL} > 1$, $r = .45$, $p < .05$) and tended to be later when the NS bout started less than 12 hr after the DS bout (i.e., $\psi_{DS-NS} < 12$, $r = .40$, $p = .08$). In addition, α on the day of release tended to be longer in animals with a NS component phased closer to the entraining lights-off transition (NS $\psi_{L/D}$, $r = .42$, $p = .06$).

Discussion

LDLD induces bimodal activity rhythms that reflect the temporal reorganization of oscillators within the central pacemaker controlling behavior. Findings supporting these conclusions include persistence of split activity entrainment under skeleton LDLD cycles (Gorman & Elliott, 2003) and bimodal patterns of SCN clock gene expression (Edelstein et al., 2003; Watanabe et al., 2007; Yan et al., in press). Split activity rhythms remain entrained

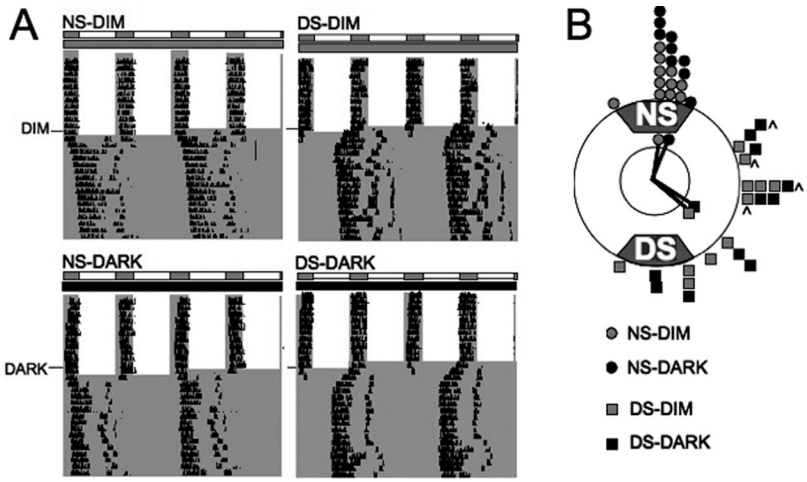

Figure 4. Fusion of 24-hr light–dark–light–dark cycle (LDLD)-induced split rhythms after release from the nighttime scotophase (NS) or daytime scotophase (DS) into constant dim illumination (DIM) or darkness (DARK) conditions in Experiment 2. (A) Representative actograms of wheel-running rhythms from animals split under LDLD with dimly lit nights, then released from the NS (left) or DS (right) into DIM (top) or DARK (bottom) conditions. Light–dark bars at the top of each actogram, along with internal shading, represent the lighting conditions in place during the experiment. (B) Phase distribution of activity onsets on the last day of LDLD. Lines represent mean angular vectors and those extending outside the inner circle indicate significant clustering for that group as determined by the Raleigh test. ^ indicates DS animals that displayed a fused rhythm continuous with the NS.

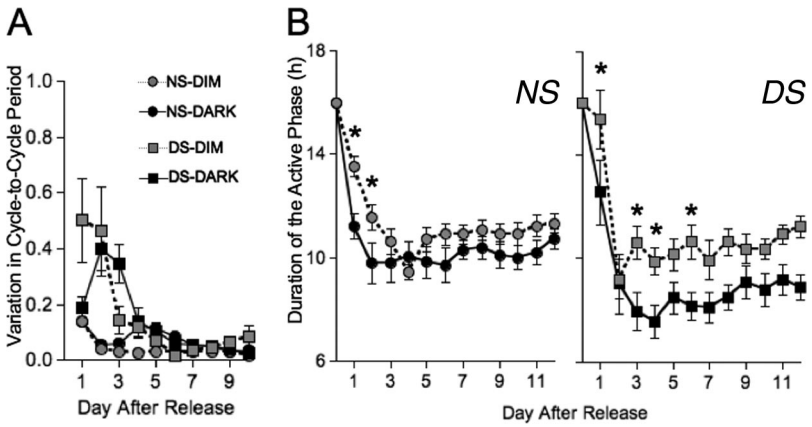

Figure 5. Quantitative measures of fusion in Experiment 2. (A) Variation in cycle-to-cycle period after release, as measured by a 3-day moving window (number of days after release represents first day in this 3-day moving window). (B) Changes in cycle-to-cycle duration of the active phase. * Least Squared means contrasts, $p < .05$. NS = nighttime scotophase; DS = daytime scotophase.

under LDLD because the two light phases counter the interactions between oscillators that would otherwise cause the split activity bouts to rejoin. Despite this action of light, the two oscillators appear to interact in a phase-dependent manner even under LDLD such that their entrainment is affected by their temporal proximity to one another (Gorman & Steele, 2006). By examining the rejoining kinetics of the split rhythms after release into constant conditions, we are able draw several novel inferences, as further elaborated below: that the two oscillators interact from the time of release into constant conditions, that the interactions are reciprocal but unequal in strength, and that dim light modulates coupling during fusion. Because entrainment of the LDLD split system is under strong experimental control, the model developed here is ideally suited for use in the further characterization of formal and physiological mechanisms governing oscillator coupling.

In making the case that release from LDLD to DD may be used as a model for understanding oscillator interactions, it is instructive to first consider the alternative—namely, that underlying oscillators have no influence on one another and that their phase is determined solely by their prior entrained phase and free-running period—an arrangement well documented in the lateralized ocular pacemakers of some invertebrate species (Roberts et al., 1987; Tomioka et al., 1991). If two oscillators have similar periods, their respective phase angle will be maintained under constant conditions, whereas if their periods differ, noninteracting oscillators will beat in and out of phase (Hudson & Lickey, 1980). The evidence against this hypothesis in the LDLD split hamster is unambiguous: Despite the clear identification of two distinct bouts of activity following release into DD, the two activity bouts do not maintain an antiphase relationship, nor do they express stable but distinct free-running periods. Instead, their fusion indicates that the underlying oscillators do interact with one another. But at what point after release into constant conditions does this happen? If initially there is no interaction between split oscillators, then the rate and pattern of fusion should be independent of release phase. NS and DS release did produce different patterns of fusion, suggesting that there is an interaction early after release into constant conditions.

Given that the two oscillators must be interactive, we can evaluate specific models for their interaction. Perhaps the simplest case would be that the two oscillators are identical in all respects, again with precedence in the invertebrate literature describing strongly coupled redundant pacemakers in visual structures. The same functional redundancy has been suggested for the left and right SCN (Donaldson & Stephan, 1982; Pickard & Turek, 1983) that correspond to LL-induced split oscillators (de la Iglesia et al., 2000; Ohta et al., 2005; Yan et al., 2005). Indeed, there is already evidence of *functional* similarities in the LDLD split paradigm: For example, either component can quickly become activity onset of the fused rhythm if one of the two daily photophases is permanently replaced with darkness (Gorman & Steele, 2006). In the same study, LDLD entrainment parameters did not differ for nighttime versus daytime bouts but instead varied only according the phase angle between them. In the present Experiment 2, the identical oscillator hypothesis is clearly contradicted by the differences in entrainment between the two activity bouts. Nevertheless, it could still be the case that the interactions between oscillators are identical, which we assessed by examining fusion following release from each scotophase. If the coupling is indeed functionally identical, then the difference between the two groups of our first experiment is an additional half cycle of entrainment when rejoining is allowed to begin. Consequently, the steady state phase of the rejoined system should vary by the time separating the two release times—12 hr—which was not the outcome. Thus, the underlying oscillators are interactive but not identical.

Differences in the coupling between oscillators may be quantitatively rather than categorically determined. Thus far, we have characterized split entrainment in LDLD as the output of two distinct oscillators, but it is unlikely that they are discrete and indivisible entities. Rather, splitting may be just one rearrangement of a population of circadian oscillators, which is commonly, but not inevitably, split more or less evenly into two groups. Previous studies, using other LDLD cycles and/or scheduled novel wheel running to attain a similar split entrainment configuration, have demonstrated reciprocity between activity bouts, suggesting a continuous distribution in the size or strength of two oscillator groups (Evans & Gorman, 2002; Gorman & Lee, 2001). Thus, possible interpretations of asymmetric entrainment under LDLD 8:4:8:4 (Experiment 2; Gorman, 2001; Gorman & Elliott, 2003; Gorman

& Steele, 2006) are that relatively more or higher amplitude oscillators are entrained to the DS. Accordingly, if the coupling interaction were proportional to the size (BL) of the activity bout, prior entrainment asymmetry should predict the rejoining dynamic, but it did not: Despite symmetry of entrainment in Experiment 1 and asymmetry in Experiment 2, the dependence of the pattern and rate of fusion on release phase was remarkably consistent across experiments.

In both experiments, the daily phase of the daytime component was more labile after release from LDLD. After both NS and DS release, fusion was typically accomplished via large advances in the phase of the daytime component regardless of whether this component was continuous with activity onset (DS release) or activity offset (NS release) of the fused rhythm. Even in cases in which the onset of the fused rhythm was continuous with the DS, the phase of the daytime component shifted more and the retrojected phase diverged considerably from the phase of the DS. Further, variance in circadian period was lower after NS release, suggesting that the phase and period of the nighttime component are determined more by its intrinsic properties than by coupling to the DS oscillator. Correlations between split entrainment measures and fusion do indicate a modest relationship between the length (BL) of the daytime component and the phase of the rejoined rhythm after NS release, suggesting that the phase of the nighttime component can be influenced by the daytime component when the system is asymmetrically split under LDLD. Taken together, these observations suggest that interactions between oscillators split by LDLD are reciprocal but not equal in strength.

For a coupled multi-oscillator system to achieve stability of rhythm waveform and period, the oscillator coupling must be phase dependent (Daan & Berde, 1978; Pittendrigh & Daan, 1976), which implies the existence of a phase response curve for coupling. Indeed, if one oscillator uniformly sped up or slowed down another oscillator regardless of their phase relationship, their periods would not converge to a common value. Phase-dependent interactions have been productively explored in invertebrate models (Page, 1981; Page & Nalovic, 1992; Tomioka, 1993; Tomioka et al., 1991; Wiedenmann, 1983), but not in mammals for lack of good analytic paradigms. At present, we have only scant data on coupling influence as a function of phase angle in LDLD split rhythms, but available evidence suggests that attraction between oscillators is minimized as their phase angle increases (Gorman & Steele, 2006) and photophase deletion studies have indicated that one oscillator influences the phase of the other during the first few hours of the intervening photophase (Gorman & Steele, 2006). Thus, following release into constant conditions, the rate and pattern of fusion is likely dictated by reciprocal but unequal interactions that are dependent on oscillator phase relationships.

A disproportionate influence of the nighttime component on the daytime component may also account for the salient observation in both experiments that retrojected phase is more variable after DS release than NS release. We may analogize the resolving of a phase discrepancy between two unequal oscillators to the resolving of a punching contest between two unevenly matched boxers. If the stronger Boxer A punches first, analogous to NS release, the fight ends immediately at a time determined by A (or "nighttime component"). However, analogous to DS release, if the weaker Boxer B ("daytime component") punches first, there is no knockout and the match continues. Affected by the blow from B, Boxer A's strength is diminished and she/he may or may not score a knockout, but the stronger punches from A lead certainly to further weakening of B. As more blows are exchanged, B's chances fade. In other words, Boxer A will usually win, but it takes longer and the outcome is more variable if the initial punch puts the combatants temporarily on more even terms. Analogously, when the nighttime component gets the first shot, being first released into darkness, rejoining follows predictably. In this scenario, the daytime component influences the rejoining kinetic initially after DS release, and this introduces variability into the steady-state outcome by diminishing the determinative influence of the nighttime component. This proposed iterative interaction of two oscillators is not a novel idea, but is rather a central feature of Daan and Berde's (1978) classic two-oscillator model. Formalizing and rigorous testing of this model will require incorporating quantitative estimates of phase-dependent oscillator coupling. Suitable coupling coefficients might be derived from studying release into constant conditions after entrainment to a variety of LDLD conditions.

Having demonstrated large effects of dim nocturnal illumination on circadian entrainment of hamsters, we argued for an action on circadian coupling (Gorman et al., 2006). In other species too, very low levels of light (e.g., 0.1–1 lux) have been suggested to modulate oscillator coupling (Meijer et al., 1990; Tomioka, 1993). Given that Experiment 1 indicated that fusion is mediated by oscillator interactions, we hypothesized that dim light may influence the rate and pattern of rejoining under constant conditions. If dim illumination were to globally inhibit interactions in the present species, we predicted that fusion would be faster if the system were released into constant darkness rather than constant dim light. On the other hand, if dim illumination were to generally strengthen interactions, we predicted that fusion would be slower after release into constant darkness. Contrary to these predictions, the same dim illumination shown to be markedly efficacious under other coupling paradigms did not alter the retrojected phase of the fused rhythm or the overall pattern of rejoining after either release phase. Constant dim illumination, however, did affect activity duration (α) of the fused rhythm in a manner that depended on the phase of release (cf. Figure 5B). Moreover, consistent with a prior study of unsplit Syrian hamsters released into constant conditions (Evans, Elliott, & Gorman, 2007), dim light lengthened the free-running period of the fused rhythm (see Table 2).

LDLD-induced splitting, fusion of split components, and the steady state of the fused rhythm all likely reflect interactions between underlying oscillators. If this is indeed the case, why do we observe differences in the effects of dim light across these three paradigms? It may be that the potent effects of dimly lit nights documented under entrained conditions derive from an interaction with the effects of brighter light during the photophase. However, this is unlikely to be the sole mechanism because dim light modulates circadian waveform under free-running conditions in which brighter light is absent (Evans et al., 2007). Another hypothesis may be that these three behavioral models do not index identical coupling mechanisms and dim light is able to influence one type of interaction but not all types. For example, dim light may be able to modulate only those coupling processes that manifest gradually. That dim light was able to modulate circadian waveform much more after DS release may indicate that when fusion is slower, the influence of dim light is easier to detect. Alternatively, it is possible that a common mechanism underlies

coupling in these diverse paradigms, but that these paradigms reflect interactions between groups of oscillators while in uniquely different phase relationships. Dim illumination may change the shape of the phase response curve for coupling such that its effects are more evident in some phase relations than in others (e.g., DS vs. NS release).

In summary, fusion of LDLD-induced split rhythms provides a useful assay of oscillator interactions. By varying the phase of release, we assessed whether the underlying coupling mechanisms operate in a component- and/or phase-dependent manner. Differences in the pattern of fusion at the behavioral level indicate that there are functional differences between split oscillator components and that there is an asymmetry in their relative influence over the other. Further, we propose that the presently split oscillators interact via phase-dependent mechanisms and that dim light modulates these interactions. Probing oscillator interactions under admittedly unecological conditions may help define coupling processes relevant to natural circadian plasticity (e.g., seasonality) and may suggest approaches for therapeutic manipulation of human circadian clocks.

References

Aton, S. J., & Herzog, E. D. (2005). Come together, right now: Synchronization of rhythms in a mammalian circadian clock. *Neuron, 48,* 531–534.

Batschelet, E. (1981). *Circular statistics in biology.* New York, NY: Academic Press.

Daan, S., & Berde, C. (1978). Two coupled oscillators: Simulations of the circadian pacemaker in mammalian activity rhythms. *Journal of Theoretical Biology, 70,* 297–313.

de la Iglesia, H. O., Cambras, T., Schwartz, W. J., & Diez-Noguera, A. (2004). Forced desynchronization of dual circadian oscillators within the rat suprachiasmatic nucleus. *Current Biology, 14,* 796–800.

de la Iglesia, H. O., Meyer, J., Carpino, A., Jr., & Schwartz, W. J. (2000). Antiphase oscillation of the left and right suprachiasmatic nuclei. *Science, 290*(5492), 799–801.

Donaldson, J. A., & Stephan, F. K. (1982). Entrainment of circadian rhythms: Retinofugal pathways and unilateral suprachiasmatic nucleus lesions. *Physiology and Behavior, 29,* 1161–1169.

Edelstein, K., de la Iglesia, H. O., & Mrosovsky, N. (2003). Period gene expression in the suprachiasmatic nucleus of behaviorally decoupled hamsters. *Molecular Brain Research, 114,* 40–45.

Evans, J. A., Elliott, J. A., & Gorman, M. R. (2005). Circadian entrainment and phase resetting differ markedly under dimly illuminated versus completely dark nights. *Behavioural Brain Research, 162,* 116–126.

Evans, J. A., Elliott, J. A., & Gorman, M. R. (2007). Circadian effects of light no brighter than moonlight. *Journal of Biological Rhythms, 22,* 356–367.

Evans, J. A., & Gorman, M. R. (2002). Split circadian rhythms of female Syrian hamsters and their offspring. *Physiology and Behavior, 76,* 469–478.

Gorman, M. R. (2001). Exotic photoperiods induce and entrain split circadian activity rhythms in hamsters. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology, 187,* 793–800.

Gorman, M. R., & Elliott, J. A. (2003). Entrainment of 2 subjective nights by daily light:dark:light:dark cycles in 3 rodent species. *Journal of Biological Rhythms, 18,* 502–512.

Gorman, M. R., Elliott, J. A., & Evans, J. A. (2003). Plasticity of hamster circadian entrainment patterns depends on light intensity. *Chronobiology International, 20*(2), 233–248.

Gorman, M. R., Evans, J. A., & Elliott, J. A. (2006). Potent circadian effects of dim illumination at night in hamsters. *Chronobiology International, 23*(1), 245–250.

Gorman, M. R., & Lee, T. M. (2001). Daily novel wheel running reorganizes and splits hamster circadian activity rhythms. *Journal of Biological Rhythms, 16,* 541–551.

Gorman, M. R., & Steele, N. A. (2006). Phase angle difference alters coupling relations of functionally distinct circadian oscillators revealed by rhythm splitting. *Journal of Biological Rhythms, 21,* 195–205.

Hudson, D. J., & Lickey, M. E. (1980). Internal desynchronization between two identified circadian oscillators in Aplysia. *Brain Research, 183,* 481–485.

Klein, D. C., Moore, R. Y., & Reppert, S. M. (Eds.). (1991). *Suprachiasmatic nucleus: The mind's clock.* New York, NY: University Oxford Press.

Kunz, H., & Achermann, P. (2003). Simulation of circadian rhythm generation in the suprachiasmatic nucleus with locally coupled self-sustained oscillators. *Journal of Theoretical Biology, 224*(1), 63–78.

Lee, H. S., Billings, H. J., & Lehman, M. N. (2003). The suprachiasmatic nucleus: A clock of multiple components. *Journal of Biological Rhythms, 18,* 435–449.

Meijer, J. H., Daan, S., Overkamp, G. J., & Hermann, P. M. (1990). The two-oscillator circadian system of tree shrews (*Tupaia belangeri*) and its response to light and dark pulses. *Journal of Biological Rhythms, 5,* 1–16.

Michel, S., & Colwell, C. S. (2001). Cellular communication and coupling within the suprachiasmatic nucleus. *Chronobiology International, 18*(4), 579–600.

Moore, R. Y., Speh, J. C., & Leak, R. K. (2002). Suprachiasmatic nucleus organization. *Cell and Tissue Research, 309,* 89–98.

Ohta, H., Yamazaki, S., & McMahon, D. G. (2005). Constant light desynchronizes mammalian clock neurons. *Nature Neuroscience, 8,* 267–269.

Page, T. L. (1981). Effects of localized low-temperature pulses on the cockroach circadian pacemaker. *American Journal of Physiology, 240*(3), R144–150.

Page, T. L., & Nalovic, K. G. (1992). Properties of mutual coupling between the two circadian pacemakers in the eyes of the mollusc Bulla gouldiana. *Journal of Biological Rhythms, 7,* 213–226.

Pickard, G. E., & Turek, F. W. (1983). The suprachiasmatic nuclei: Two circadian clocks? *Brain Research, 268*(2), 201–210.

Pittendrigh, C. S., & Daan, S. (1976). A functional analysis of circadian pacemakers in nocturnal rodents: V. Pacemaker structure: A clock for all seasons. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology, 106,* 333–355.

Roberts, M. H., Block, G. D., & Lusska, A. E. (1987). Comparative studies of circadian pacemaker coupling in opisthobranch molluscs. *Brain Research, 423,* 286–292.

Shinohara, K., Honma, S., Katsuno, Y., Abe, H., & Honma, K. (1995). Two distinct oscillators in the rat suprachiasmatic nucleus in vitro. *Proceedings of the National Academy of Sciences of the USA, 92,* 7396–7400.

Strogatz, S. H. (2003). *Sync: The emerging science of spontaneous order.* New York, NY: Hyperion.

Tomioka, K. (1993). Analysis of coupling between optic lobe circadian pacemaker in the cricket *Gryllus bimaculatus. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology, 172,* 401–408.

Tomioka, K., Yamada, K., Yokoyama, S., & Yoshihiko, C. (1991). Mutual interactions between optic lobe circadian pacemaker in the cricket *Gryllus bimaculatus. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology, 169,* 291–298.

Watanabe, T., Naito, E., Nakao, N., Tei, H., Yoshimura, T., & Ebihara, S. (2007). Bimodal clock gene expression in mouse suprachiasmatic nucleus and peripheral tissues under a 7-hour light and 5-hour dark schedule. *Journal of Biological Rhythms, 22*(1), 58–68.

Welsh, D. K., Logothetis, D. E., Meister, M., & Reppert, S. M. (1995).

Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. *Neuron, 14,* 697–706.

Wiedenmann, G. (1983). Splitting in a circadian activity rhythm: The expression of bilaterally paired oscillators. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology, 150,* 51–60.

Yamaguchi, S., Isejima, H., Matsuo, T., Okura, R., Yagita, K., Kobayashi, M., & Okamura, H. (2003). Synchronization of cellular clocks in the suprachiasmatic nucleus. *Science, 302*(5649), 1408–1412.

Yan, L., Foley, N. C., Bobula, J. M., Kriegsfeld, L. J., & Silver, R. (2005). Two antiphase oscillations occur in each suprachiasmatic nucleus of behaviorally split hamsters. *Journal of Neuroscience, 25,* 9017–9026.

Yan, L., Silver, R., & Gorman, M. R. Reorganization of suprachiasmatic nucleus networks under 24 h LDLD conditions. *Journal of Biological Rhythms,* in press.

Received July 28, 2009
Revision received October 1, 2009
Accepted October 2, 2009 ■