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# Dim Nighttime Illumination Interacts With Parametric Effects of Bright Light to Increase the Stability of Circadian Rhythm Bifurcation in Hamsters

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The endogenous circadian pacemaker of mammals is synchronized to the environmental day by the ambient cycle of relative light and dark. The present studies assessed the actions of light in a novel circadian entrainment paradigm where activity rhythms are bifurcated following exposure to a 24-h light:dark:light:dark (LDLD) cycle. Bifurcated entrainment under LDLD reflects the temporal dissociation of component oscillators that comprise the circadian system and is facilitated when daily scotophases are dimly lit rather than completely dark. Although bifurcation can be stably maintained in LDLD, it is quickly reversed under constant conditions. Here the authors examine whether dim scotophase illumination acts to maintain bifurcated entrainment under LDLD through potential interactions with the parametric actions of bright light during the two daily photophases. In three experiments, wheel-running rhythms of Syrian hamsters were bifurcated under LDLD with dimly lit scotophases, and after several weeks, dim scotophase illumination was either retained or extinguished. Additionally, "full" and "skeleton" photophases were employed under LDLD cycles with dimly lit or completely dark scotophases to distinguish parametric from nonparametric effects of bright light. Rhythm bifurcation was more stable in full versus skeleton LDLD cycles. Dim light facilitated the maintenance of bifurcated entrainment under full LDLD cycles but did not prevent the loss of rhythm bifurcation in skeleton LDLD cycles. These studies indicate that parametric actions of bright light maintain the bifurcated entrainment state; that dim scotophase illumination increases the stability of the bifurcated state; and that dim light interacts with the parametric effects of bright light to increase the stability of rhythm bifurcation under full LDLD cycles. A further understanding of the novel actions of dim light may lead to new strategies for understanding, preventing, and treating chronobiological disturbances. (Author correspondence: jevans@msm.edu)

**Keywords:** Bifurcated rhythms, Circadian coupling, Oscillator interactions, Parametric and nonparametric entrainment, Plasticity, Splitting

## INTRODUCTION

In mammals, the suprachiasmatic nucleus (SCN) programs daily rhythms in behavior and physiology so that they are synchronized to the sidereal day (Klein et al., 1991;Weaver, 1998). Lighting changes across the day are the primary cue synchronizing circadian rhythms in mammals (Meijer & Schwartz, 2003), and rapid change in photic irradiance during twilight transitions is a reliable indicator for phase of the solar cycle (Roenneberg & Foster, 1997). Under laboratory conditions, photoentrainment in nocturnal rodents can be maintained under "skeleton" photoperiods that replace the full photophase with two short light pulses bracketing subjective night (Pittendrigh & Daan, 1976). These findings generally support a "nonparametric" model of photoentrainment whereby phase-resetting actions of light at dawn and dusk are critical for entrainment (Aschoff, 1960; Johnson et al., 2003; Pittendrigh, 1981). However, entrainment is not stable when skeleton photoperiods simulate longer photophases (>12 h), and a "phase jump" will occur beyond a "minimum tolerable night," wherein activity abruptly crosses one of the entraining light pulses and realigns into the longer scotophase (Pittendrigh & Daan, 1976; Sharma et al., 1997; Stephan, 1983). Full photophases prevent phase jumps and thus are viewed as exerting "parametric" actions that stabilize entrainment under very long day lengths. Parametric effects of light are also evident under constant conditions where increasing light intensity, for example, generally lengthens the period of the free-running rhythm in nocturnal rodents (Aschoff, 1979).

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A cardinal feature of the circadian visual system, derived from extensive quantification of nonparametric responses (e.g., phase resetting), is that it is characterized by a higher threshold than the classical image-forming visual system (Meijer & Schwartz, 2003; Nelson & Takahashi, 1991; Takahashi et al., 1984). These findings notwithstanding, circadian function can be influenced in a pronounced manner by light that is below the threshold for eliciting these classic circadian responses (Chiesa et al., 2006; Gorman et al., 2006). For example, re-entrainment after a shift in the light:dark cycle is accelerated when the scotophase is dimly illuminated (e.g., with green light of an irradiance no greater than that of dim moonlight) rather than completely dark (Evans et al., 2009; Frank et al., 2010). With the same scotophase manipulation, the range of entrainment to light:dark cycles with a period greater than 24 h is increased, as is the rate of short-photoperiod adaptation (e.g., expansion of activity time following transfer from longer photoperiods; Gorman & Elliott, 2004; Gorman et al., 2006). These robust effects occur despite the fact that the phase-resetting actions of bright light pulses are not enhanced against a background of dim illumination versus complete darkness (Evans et al., 2007).

Among the robust effects of dim scotophase illumination is that it facilitates bifurcated entrainment under 24-h light:dark:light:dark (LDLD) cycles (Gorman et al., 2003). Rather than consolidate activity into one scotophase, hamsters and mice that display bifurcated entrainment under LDLD divide their activity between the two daily scotophases (Gorman & Elliott, 2003), reflecting the temporal dissociation of component circadian oscillators in the SCN (Watanabe et al., 2007; Yan et al., 2010). Rhythm bifurcation under LDLD occurs in nearly all animals when daily scotophases incorporate dim illumination comparable in intensity to dim moonlight. For example, bifurcated entrainment occurs in nearly 100% of Syrian hamsters held under LDLD with dimly lit nights, compared to only 25–33% housed with completely dark nights (Evans et al., 2005; Gorman et al., 2003). Whereas the actions of dim scotophase illumination in promoting induction of bifurcated rhythms in LDLD have been well described (Evans et al., 2005), it remains undetermined whether, once so entrained, dim light is required for the maintenance of bifurcated rhythms.

Once bifurcated entrainment is induced under LDLD, the bright daily photophases keep the two activity bouts entrained within their respective scotophases. Conversely, activity components rejoin under the influence of mutual oscillator coupling within just a few circadian cycles in constant dim or dark conditions (Evans et al., 2010). Nonparametric actions of bright light (i.e., resetting by light pulses at the beginning and end of subjective night) are at least sometimes sufficient to maintain bifurcated rhythms, since this pattern of entrainment persists in Syrian and Siberian hamsters held under "skeleton" LDLD cycles (i.e., a total of four 1-h pulses/24 h), where two short light pulses replace each of the two 7-h photophases (Gorman & Elliott, 2003) or 9-h photophases (Rosenthal et al., 2005). In contrast, bifurcated rhythms of mice readily rejoin under skeleton LDLD cycles, establishing that parametric actions of light during the full photophases of LDLD contribute to bifurcated entrainment in that species (Gorman & Elliott, 2003). Even for hamsters, however, nonparametric actions of light are insufficient to explain the systematic variation in entrainment parameters that is observed following manipulation of photophase length in the bifurcated system (Gorman & Steele, 2006).

As is the case in many dynamical control systems, alternative circadian states may be stable under a given set of environmental conditions, and the history of the system may strongly influence its response to environmental conditions (i.e., the system exhibits hysteresis). Having previously established an important role of dim light in the induction of rhythm bifurcation in LDLD (Evans et al., 2005), we ask here whether dim light is necessary for its maintenance with a view to clarifying its effects on circadian function. First, if the circadian pacemaker exhibits bistability, then the extinction of dim illumination should have no effect once rhythms have bifurcated. Second, if dim light acts to maintain rhythm bifurcation solely through an interaction with nonparametric effects of bright light, then identical effects should be observed in full and skeleton LDLD cycles. On the other hand, if dim light interacts specifically with parametric effects of bright light, then its presence should influence results obtained under full, but not skeleton, LDLD conditions. In three experiments, bifurcated entrainment was induced in Syrian hamsters held under LDLD with full photophases and dimly lit scotophases. After several weeks, dim scotophase illumination was either retained or extinguished under a full or skeleton LDLD cycle. Dim light was critical for maintaining rhythm bifurcation in full LDLD cycles, but did not prevent loss of rhythm bifurcation in skeleton LDLD cycles. The results indicate that dim scotophase illumination alters the stability of the bifurcated state; that parametric actions of bright light promote the maintenance of rhythm bifurcation; and that dim light affects the parametric influences of bright light on circadian rhythmicity.

## MATERIALS AND METHODS

Syrian hamsters (*Mesocricetus auratus*) were bred from stock originally purchased from Harlan (HsdHan; AURA, Indianapolis, IN) and raised under a 14 h light:10 h dark cycle (LD14:10; lights-on: 03:00 h Pacific Standard Time [PST], lights-off: 17:00 h PST; photophase: 50–75 lux, scotophase: 0 lux). After weaning, animals were group-housed without running wheels inside polypropylene cages (48 cm × 27 cm × 20 cm) in a room where ambient temperature was maintained at 22°C ± 2°C, with ad libitum access to water and food (Purina Rodent Chow no. 5001, St Louis, MO). For each of three

experiments, hamsters (6–8 wks of age) were transferred to individual wheel-running cages at the start of a new LDLD7:5:7:5 photocycle (lights-on: 03:00 h PST, lights-off: 10:00 h PST, lights-on: 15:00 h PST, lights-off: 22:00 h PST) with dim scotophase illumination (see below), coincident with the start of the daytime scotophase (10:00 h PST). Under LDLD, broad-spectrum, cool white fluorescent bulbs provided photophase illumination of 70–110 lux (Experiments 1–2) or 200–300 lux (Experiment 3).

In each experiment, the independent variable was the presence or absence of dim illumination during daily scotophases. At the beginning of LDLD7:5:7:5, both the daytime scotophase (DS) and nighttime scotophase (NS) were marked by dim scotophase illumination (<0.1 lux, equivalent to $<1.79 \times 10^{-8}$ W/cm$^2$ and $<5.1 \times 10^{10}$ photons/cm$^2$·s) provided to each animal by a narrow bandwidth green light-emitting diode (peak $\lambda$ = 560 nm with a half bandwidth of 23 nm) mounted externally and facing the back wall of the cage. Several weeks after transfer, the light-emitting diodes (LEDs) were either extinguished (DARK-) or retained (DIM-) under LDLD with "full" 7-h photophases (fLDLD, same as the initial LDLD cycle to which animals were first transferred), or a "skeleton" LDLD cycle (sLDLD) where daily photophases were simulated using 1-h light pulses bracketing each of the daily scotophases (Figure 1). The sLDLD is equivalent to a LD1:5 cycle in which each 1-h light pulse is followed by 5 h of relative darkness.

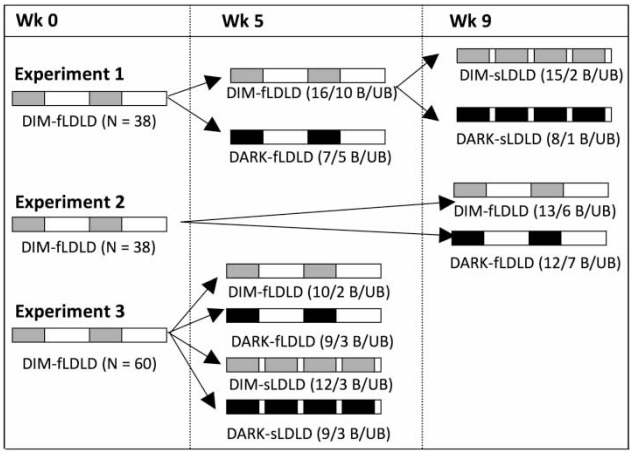


FIGURE 1. Schematic representation of protocols for Experiments 1–3. Light:dark bars represent the 24-h photoperiods, singly plotted, to which animals were exposed at the end of the week indicated. Following 5 or 9 full weeks of initial exposure to DIM-fLDLD, bifurcated (B) and unbifurcated (UB) hamsters continued in LDLD cycles with "full" 7-h photophases (fLDLD) or "skeleton" photophases (sLDLD), and scotophases with either dim nighttime illumination (DIM-) or complete darkness (DARK-). The number of bifurcated and unbifurcated hamsters in each group at the time of experimental manipulation is noted below each lighting cycle. Note that these do not represent denominators for subsequent percentage plots, because hamsters may have subsequently rejoined or rebifurcated in the ensuing interval.

All procedures were approved by the Institutional Animal Care and Use Committee of the University of California, San Diego, and conformed to international standards for animal research (Portaluppi et al., 2010). To minimize the number of litters required for these experiments, equal numbers of male and female offspring were assigned to each study. Because there were no sex differences evident in any statistical analyses, all results presented are collapsed across sex. Cage changes were scheduled judiciously at specific times during the experiment. During the first 5 wks under LDLD (i.e., before scotophase or photophase manipulations), cage changes were scheduled in a manner designed to facilitate rhythm bifurcation (Evans et al., 2005). Cage changes occurred during the first 90 min of the daytime scotophase under the direction of a dim red headlamp (<2 min exposure/animal) either 1 wk (Experiments 1–2) or 2 wks (Experiment 3) after transfer to LDLD. Thereafter, cages were changed every 2–3 wks either at the same phase in dim red light (Experiments 1–2) or during the afternoon photophase (Experiment 3). Cage changes were not performed for 2 wks after experimental manipulations.

### Experiment 1

Hamsters (n = 38, 19/sex) were transferred to individual wheel-running cages (27 cm × 20 cm × 15 cm) inside light-tight environmental chambers (1 cage/chamber). After 5 wks of DIM-fLDLD, the dim scotophase illumination was either retained (DIM-fLDLD; n = 26) or extinguished at the beginning of the nighttime scotophase (Figure 1; DARK-fLDLD; n = 12). After 9 wks under DIM-fLDLD, animals were transferred to skeleton LDLD cycles with either dimly illuminated (DIM-sLDLD; n = 17) or dark scotophases (DARK-sLDLD; n = 9). Skeleton light pulses were initiated immediately after the 5-h nighttime scotophase. Animals remained under their respective conditions for 4 wks.

To ensure compatible representation of bifurcated and unbifurcated rhythms within experimental groups at the start of each manipulation, animals were assigned to conditions in a manner that matched animals within groups based on behavioral state (i.e., bifurcation or unbifurcated) and the number of days in that behavioral state. In Experiment 1, unequal samples were constructed for DIM-fLDLD and DARK-fLDLD so that the subsequent sLDLD manipulation could be performed using only DIM-fLDLD animals with adequate sample sizes.

### Experiment 2

Because Experiment 1 demonstrated effects of dim illumination in fLDLD cycles at Week 5 and no effect in sLDLD cycles at Week 9, Experiment 2 was conducted to assess whether dim illumination would maintain bifurcated rhythms in fLDLD cycles after 9 wks (Figure 1). Hamsters (n = 38, 19/sex) were transferred to DIM-fLDLD as in Experiment 1. After 9 wks, the LEDs were either retained (DIM-fLDLD, n = 19) or extinguished at

the beginning of the nighttime scotophase (DARK-fLDLD, n = 19). As in Experiment 1, subjects were assigned to experimental conditions to ensure comparable representation within each experimental group at the start of the manipulation. Animals remained under their respective conditions for 4 wks.

### Experiment 3

Experiments 1 and 2 suggested that the effects of dim illumination depended on the presence of full versus skeleton photophases under LDLD. To assess directly whether dim scotophase illumination affected rhythm bifurcation differently in fLDLD versus sLDLD cycles, Experiment 3 manipulated these two variables simultaneously in a 2 × 2 design (Figure 1). Alternate housing conditions were used in this final experiment to permit simultaneous fLDLD and sLDLD manipulations with adequate sample sizes. Hamsters (n = 60, 30/sex) were transferred to larger wheel-running cages (48 cm × 27 cm × 20 cm) inside light-tight environmental chambers (12 cages/chamber) coincident with the daytime scotophase of a LDLD7:5:7:5 cycle like that employed in Experiments 1 and 2. After 5 wks under DIM-fLDLD, hamsters were assigned to one of four groups, which differed by (1) whether the daily scotophases were DIM- or DARK- and (2) whether the LDLD cycle was fLDLD or sLDLD (Figure 1; DIM-fLDLD: n = 10, DARK-fLDLD: n = 9, DIM-sLDLD: n = 12, DARK-sLDLD: n = 9). Due to space constraints, nine unbifurcated hamsters were not assigned to an experimental condition and were excluded from the study. Scotopic manipulations began at the start of the nighttime scotophase, and skeleton light pulses were initiated immediately after the nighttime scotophase. Animals remained under their respective conditions for 4 wks.

### Data Collection and Analyses

For all three experiments, entrainment parameters of bifurcated rhythms were calculated during the 5th and the 9th wk under DIM-fLDLD. Activity onset for each activity bout was identified for each day of the analysis interval as the first 6-min bin above a threshold value of 15 counts/min followed by two consecutive bins above threshold (across experiments, the mean counts/min over the 24 h day was 24.55, with a standard deviation of ±9.6 counts/min). 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 ($NS_{BL}$, $DS_{BL}$). To assess whether activity under DIM-fLDLD was symmetrically bifurcated, the average ratio of the NS and DS bout length ($NS_{BL}/DS_{BL}$) was calculated. Additionally, the phase angle between bifurcated activity bouts was determined from the average number of hours between the onset of the NS activity bout and that of the DS activity bout ($\psi_{NS\text{-}DS}$).

To determine the effects of dimly lit nights and completely dark nights on the maintenance of bifurcated rhythms under fLDLD and sLDLD, qualitative analyses categorized animals into groups based on whether bifurcated entrainment was maintained during the 4 wks subsequent to each experimental manipulation. Animals that had not bifurcated within the first 5 wks under DIM-fLDLD were not considered in subsequent analyses. Consistent with previous experiments (Evans et al., 2005; Gorman et al., 2003), activity rhythms were categorized as bifurcated if there were wheel-running bouts longer than 30 min during each of the two daily dark periods for at least 5 consecutive days, and as no longer bifurcated (i.e., rejoined) if one of the two bifurcated activity bouts disappeared for at least 5 consecutive days. Rejoining incidence was calculated with respect to the total number of animals bifurcated at the beginning of an analysis interval.

### Statistical Analyses

Statistical tests were conducted with JMP software (SAS Institute, Cary, NC). Categorical data were analyzed using contingency statistics (Pearson's $\chi^2$). To assess bifurcation symmetry, entrainment measures were tested with a one-sample $t$ test to determine whether $NS_{BL}/DS_{BL}$ was equal to 1 and whether $\psi_{NS\text{-}DS}$ was equal to 12 h. Values in text and figures are mean ± SEM unless otherwise indicated.

## RESULTS

### Experiment 1

In DIM-fLDLD, 100% of the animals adopted bifurcated activity rhythms within the first 5 wks of LDLD exposure (Figure 2A–D), but in 55% (21/38) of these animals, the bifurcated activity components rejoined at least transiently in the same interval (Figure 2C–D). In animals displaying bifurcated rhythms during the 5th wk of DIM-fLDLD, bifurcated entrainment to the LDLD cycle was asymmetric. Specifically, the NS activity bout was significantly shorter than the DS activity bout (Table 1; $t_{(22)} = -6.2$, $p < .0001$), and the NS activity bout phase-led the DS activity bout (i.e., fewer hours separated the onset of the NS activity bout from that of the next DS activity bout (mean $\psi_{NS\text{-}DS} = 9.9$ h) than vice versa (mean $\psi_{DS\text{-}NS} = 14.1$); h, Table 1; $t_{(22)} = 7.2$, $p < .0001$). Four weeks later, animals with bifurcated rhythms maintained under DIM-fLDLD displayed a NS bout that was significantly longer than the DS bout (Table 1; $t_{(23)} = 2.5$, $p = .04$) and tended to phase-lag the DS bout (Table 1; $t_{(23)} = -1.7$, $p = .09$). These systematic changes in bifurcated entrainment are evident in the behavior of individual animals (Figure 2A, E, F).

When dim scotophase illumination was extinguished after 5 wks in fLDLD, bifurcated rhythms of all DARK-fLDLD animals rejoined within the subsequent 4 wks (Figures 2B, 3A). Rejoining of bifurcated rhythms occurred among significantly fewer animals maintained under DIM-fLDLD (Figures 2A, C, 3A; $\chi^2(1) = 15.7$, $p < .0001$). When sLDLD cycles were introduced 4 wks

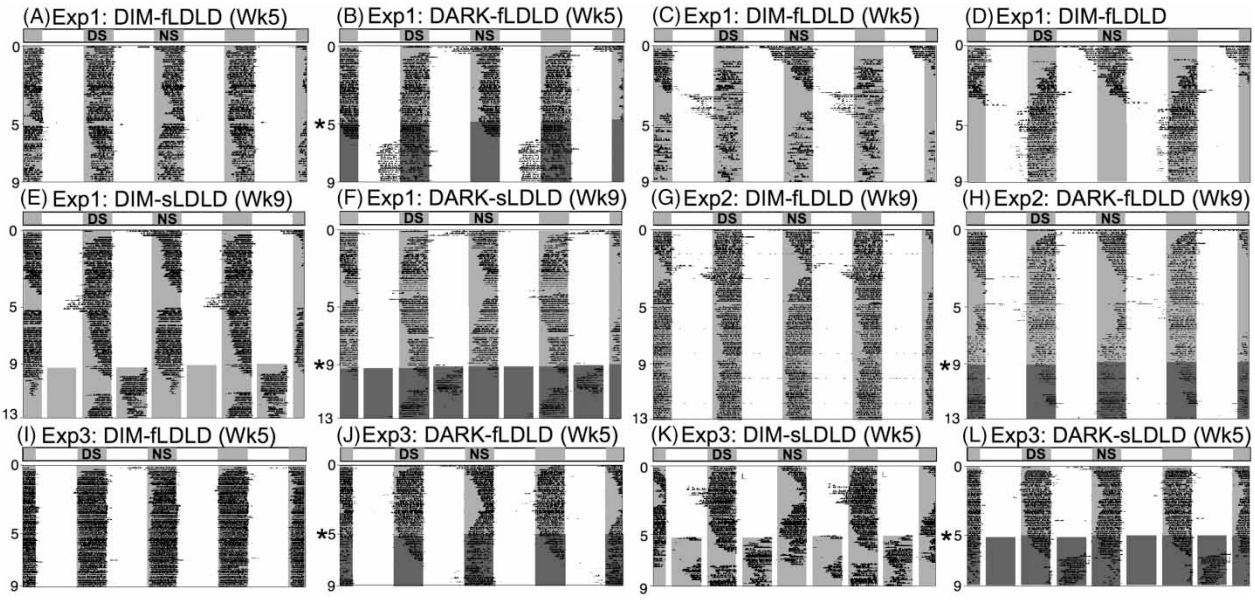


FIGURE 2. Representative double-plotted, wheel-running actograms illustrating the behavior of hamsters studied in Experiment 1 (A–F), Experiment 2 (G–H), and Experiment 3 (I–L). The light:dark bar at the top of each actogram represents the lighting condition in place for at least the first 5 wks of each experiment. After either 5 (A–D, I–L) or 9 (G–H) wks under this condition, the lighting cycle either remained the same or was altered in the manner indicated by the label for each actogram and represented by changes in the internal shading. An asterisk on the *y*-axis indicates the day of the transition from dimly lit to completely dark scotophases. DIM = dimly lit scotophases; DARK = completely dark scotophases; fLDLD = "full" LDLD cycle with 7-h photophases; sLDLD = "skeleton" LDLD cycle where daily photophases present under fLDLD were simulated using 1-h light pulses. For A, C, and D, data from wks 9–13 are not shown.

TABLE 1. Entrainment measures under DIM-fLDLD in Experiments 1-3

| Expt | Wk | $NS_{BL}$ | $DS_{BL}$ | $NS_{BL}$/ $DS_{BL}$ | $\psi$ NS-DS | n |
|---|---|---|---|---|---|---|
| 1 | 5 | 2.6 ±0.2 | 4.4 ±0.2 | 0.6 ±0.1* | 9.9 ± 0.3 h* | 23 |
| | 9 | 3.6 ±0.2 | 3.1 ±0.1 | 1.2 ± 0.1* | 12.4 ± 0.3 h# | 15 |
| 2 | 5 | 2.8 ±0.3 | 4.1 ±0.2 | 0.7 ±0.1* | 10.5 ± 0.5 h* | 13 |
| | 9 | 3.6 ±0.2 | 3.0 ±0.1 | 1.2 ± 0.1# | 12.2± 0.4 h | 14 |
| 3 | 5 | 3.6 ±0.2 | 4.6 ±0.1 | 0.8 ±0.1* | 10.9 ±0.2 h* | 41 |
| | 9 | 4.2 ± 0.2 | 3.5 ±0.3 | 1.3 ± 0.1# | 12.8 ± 0.4 h# | 10 |

DS: Daytime Scotophase
NS: Nighttime Scotophase
BL: Bout Length
$\psi$ DS-NS: Phase angle between the DS and NS
*p<0.05, #p< 0.1;Different from predicted mean if bifurcated symmetrically

later, only one animal in each scotopic condition remained bifurcated throughout sLDLD. Most animals rejoined their bifurcated activity rhythms within 1 wk of sLDLD (Figure 2E–F), and the two groups did not differ in the incidence of rejoining under sLDLD (Figure 3B; $\chi^2_{(1)} = 0.1$, $p > .7$).

## Experiment 2

Rhythms of 82% (31/38) of animals bifurcated during the first 5 wks under DIM-fLDLD (Figure 2G–H), but 61% (23/31) of animals rejoined at least transiently within this same interval (Figure 2H). Those rhythms that were bifurcated in the 5th week of DIM-fLDLD were asymmetrically entrained: As in Experiment 1, the NS activity bout was significantly shorter (Table 1; $t_{(12)} = -2.4$, $p < .05$) and phase-led the DS bout (Table 1; $t_{(12)} = 3.2$, $p < .01$). In the 9th and final weeks of DIM-fLDLD, the NS activity bout tended to be longer than the DS activity bout (Table 1; $t_{(13)} = 1.8$, $p = .09$), but the phase angle difference between bifurcated bouts was not asymmetric (Table 1; $t_{(13)} = -0.4$, $p > .6$). Considering only the subset of four animals that remained continuously bifurcated throughout the first 9 wks under DIM-fLDLD, bifurcated entrainment tended to be asymmetric at Week 5 ($NS_{BL}/DS_{BL} = 0.6 \pm 0.1$, $t_{(3)} = -3.1$, $p = .05$; $\psi_{NS\text{-}DS} = 10.0 \pm 0.7$, $t_{(3)} = 2.8$, $p = .07$), but not at Week 9 ($NS_{BL}/DS_{BL} = 1.2 \pm 0.1$, $t_{(3)} = 2.2$, $p > .1$; $\psi_{NS\text{-}DS} = 12.5 \pm 0.3$, $t_{(3)} = -1.6$, $p > .2$). For the entire sample of animals used for this experiment, eliminating dim scotophase illumination after 9 wks

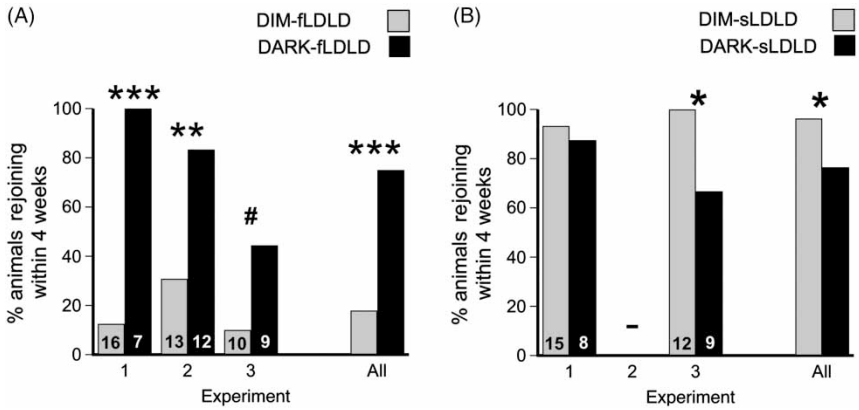


FIGURE 3. Incidence of rejoining of bifurcated rhythms under fLDLD (A) and sLDLD (B) DIM-fLDLD (gray bars) versus DARK-fLDLD (black bars) for Experiments 1–3 analyzed separately and as a composite. Note that scotopic manipulations were initiated after 5 wks under fLDLD in Experiments 1 and 3, but after 9 wks in Experiment 2. Asterisks indicate statistically significant differences between scotopic conditions (***$p < .001$; **$p < .01$; *$p < .05$; #$p < .1$). Sample size is given in bars representing each condition.

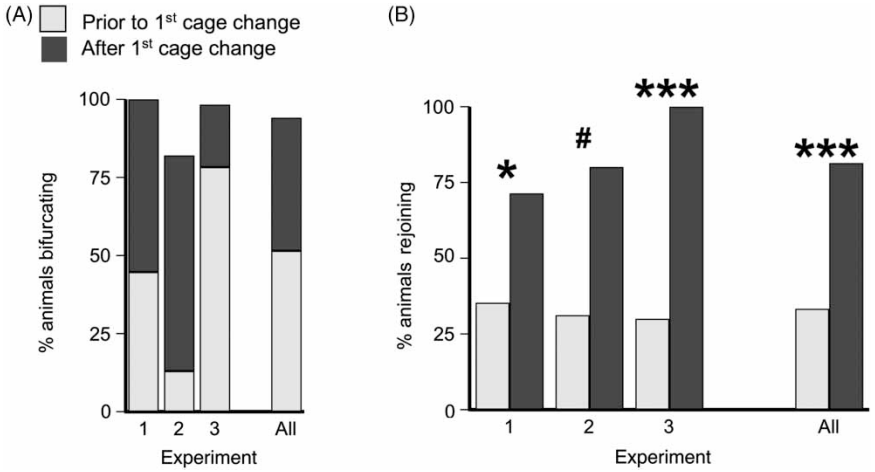


FIGURE 4. Timing of bifurcated rhythm emergence (A) influenced the incidence of spontaneous rejoining during the first 5 wks of each experiment (B). (A) In Experiments 1–3, the proportion of the initial sample that exhibited bifurcated rhythms during the first 5 wks of each experiment is divided into those animals that bifurcated before (open bars) or after the first cage change (shaded bars). (B) The incidence of spontaneous rejoining during the first 5 wks of each experiment differed for these two subsets of animals (***$p < .001$; *$p < .05$; #$p < .1$).

under fLDLD induced greater rejoining of bifurcated rhythms compared to animals maintained under DIM-fLDLD (Figures 2G–H, 3A, $\chi^2_{(1)} = 7.0$, $p < .01$).

### Experiment 3

Rhythms of 98% (59/60) of animals bifurcated within the first 5 wks of DIM-fLDLD (Figure 2I–L), and 46% (27/58) of animals rejoined at least transiently within this same interval. As in Experiments 1 and 2, the activity bouts of bifurcated rhythms were asymmetrically entrained after 5 wks in DIM-fLDLD (Table 1; $NS_{BL}/DS_{BL}$: $t_{(40)} = -3.6$, $p < .001$; $\psi_{NS\text{-}DS}$: $t_{(40)} = 5.2$, $p < .001$) and tended to be asymmetrically entrained in the opposite fashion after 9 wks in DIM-fLDLD (Table 1; $NS_{BL}/DS_{BL}$: $t_{(9)} = 2.1$, $p = .07$; Week 9 $\psi_{NS\text{-}DS}$: $t_{(9)} = -2.1$, $p = .07$). A two-factor nominal logistic analysis revealed a significant increase in rejoining in sLDLD versus fLDLD cycles ($\chi^2_{(1)} = 17.7$, $p = .0001$) and a significant interaction of this factor with scotopic condition ($\chi^2_{(1)} = 8.8$, $p = .01$), reflecting differential effects of dim scotophase illumination on the maintenance of bifurcated entrainment under fLDLD and sLDLD. Under fLDLD, dim illumination tended to preserve the bifurcated state, with fewer animals rejoining in DIM-fLDLD than in DARK-fLDLD (Figures 2I–J, 3A; $\chi^2_{(1)} = 2.9$, $p = .09$). Under sLDLD, the majority of animals in both DIM and DARK groups rejoined, but dim scotophase illumination facilitated rejoining (Figure 3B; $\chi^2_{(1)} = 4.67$, $p < .05$). Although three DARK animals remained bifurcated throughout the 4-wk exposure to sLDLD, all DIM animals rejoined during this interval. Moreover, the majority of DARK-sLDLD animals continued to display bifurcated rhythms with two activity components during the 1st wk of sLDLD (Figure 2L), but the majority of DIM-sLDLD animals displayed more than two bouts of activity during this time. One DIM-sLDLD animal initially

displayed a bout of activity in each of the four daily dark periods (Figure 2K).

### Composite Analyses

In each experiment, the vast majority of animals displayed bifurcated entrainment during the first 5 wks after the initial transfer to DIM-fLDLD (Figure 4A), but whether bifurcation occurred before or after the first cage change varied significantly between experiments ($\chi^2_{(1)} = 34.8$, $p < .0001$), as did the mean number of days until bifurcation first occurred ($F_{(2,\ 125)} = 10.98$, $p < .0001$). These differences in the timing of rhythm bifurcation predicted whether rejoining occurred spontaneously in the first 5 wks of DIM-fLDLD (Figure 4B), with a longer latency to bifurcation associated with a higher incidence of spontaneous rejoining ($\chi^2_{(1)} = 30.0$, $p < .0001$). However, the first instance of bifurcation did not differ between DIM and DARK groups ($p > .3$ for each study), indicating that this factor was effectively controlled during group assignment. Collapsing across all experiments, rejoining occurred less frequently in DIM-fLDLD than DARK-fLDLD (Figure 3A; $\chi^2_{(1)} = 21.8$, $p < .0001$), but more frequently in DIM-sLDLD than in DARK-sLDLD (Figure 3B; $\chi^2_{(1)} = 4.1$, $p < .05$). Lastly, there was no significant correlation between $\psi_{NS\text{-}DS}$ or $NS_{BL}/DS_{BL}$ and the number of days to rejoin after a scotophase or photophase manipulation in any of the three experiments when analyzed separately or when analyzed as a combined data set.

## DISCUSSION

Dynamical systems, such as the circadian pacemaker, commonly exhibit bistability and hysteresis (i.e., two stable, but path-dependent states under the same set of external conditions) (Kawato & Suzuki, 1980). As a concrete example, under free-running conditions, the rhythms of tree shrews (*Tupaia*) will split into two components when light intensity is dropped below a critical value near 1 lux (Hoffmann, 1971; Meijer et al., 1990). However, as light intensity is subsequently increased, the unsplit state is not restored until light intensity exceeds 100 lux (Hoffmann, 1971). Thus, between 1 and 100 lux, tree shrew rhythms may be stable in either a split or unsplit pattern depending on their prior circadian state. Comparable bistability is observable in a number of organisms exposed to skeleton photoperiods under which the history of light exposure and/or circadian behavioral responses determines the entrainment response to a given set of photoperiod conditions (Pittendrigh, 1966; Saunders, 1975). We addressed an analogous question with regard to the role of dim scotophase illumination and the expression of bifurcated activity rhythms of hamsters in LDLD, which has been established by both behavioral and physiological analyses to reflect the temporal dissociation of circadian oscillators within the SCN (Evans et al., 2010; Gorman & Steele, 2006; Watanabe et al., 2007; Yan et al., 2010). Across three separate studies that varied in the incidence and timing of initial rhythm bifurcation, the extinction of dim scotophase illumination increased the incidence of rejoining under fLDLD. Thus, dim scotophase illumination facilitates both the induction (Gorman et al., 2003) and maintenance of bifurcated entrainment under fLDLD cycles (present study). Consequently, we find no evidence for bistable circadian states under this paradigm. In contrast, the vast majority of animals under sLDLD rejoined whether the daily dark periods were dimly lit or completely dark. As elaborated below, this pattern of results indicates that dim scotophase illumination interacts with parametric effects of bright light on oscillator coupling to promote greater stability of the bifurcated state.

Under LDLD, the bifurcated state is maintained through the stabilizing actions of one or both of the photophases separating the two daily scotophases (Evans et al., 2010; Gorman & Elliott, 2003; Gorman & Steele, 2006). Entrainment theory has traditionally distinguished between two potential actions of light: "nonparametric" referring to the induction of discrete phase shifts by light typically falling at the beginning and end of subjective night (i.e., dawn and dusk) and "parametric" referring to continuous actions of light on the velocity of the circadian oscillation acting over long intervals (e.g., several h). These are operationalized in the present study as the effects of the 1-h skeletons versus the full 7-h photophases, respectively. The greater loss of bifurcation in sLDLD versus fLDLD conditions establishes that parametric actions of bright light promote the maintenance of bifurcated entrainment in the presence of dim scotophase illumination. The nearly universal rejoining observed in DIM-sLDLD (26/27 hamsters in Experiments 1 and 3 combined), however, greatly contrasts with a previous report in which only 1/7 hamsters rejoined under identical LD1:5 skeleton conditions (Gorman & Elliott, 2003). The nature or timing of the fLDLD to sLDLD transition may account for this discrepancy, since the present studies employed abrupt transitions, and the earlier study introduced the skeleton pulses gradually over the course of several weeks. Comparisons between studies using Siberian hamsters also suggest that gradual versus abrupt transfer to sLDLD cycles promotes greater stability (cf. Gorman & Elliott, 2003; Rosenthal et al., 2005). This implies that there are time- or history-dependent effects of entrainment to LDLD, which is confirmed by the consistent and systematic changes in bifurcated entrainment that occurred between Weeks 5 and 9 under full LDLD cycles with dimly lit nights (see Table 1). Systematic changes in bifurcated entrainment were also evident among animals in Experiment 2 that never rejoined during DIM-fLDLD.

The effect of dim scotophase illumination per se on the stability of the bifurcated state could logically derive from an interaction with either the nonparametric or parametric actions of bright light. If the primary action

was to facilitate nonparametric resetting by bright light just before and after each scotophase, then we predicted there would be no differential effect of extinguishing dim scotophase illumination in full versus skeleton LDLD cycles. The nonparametric hypothesis, thus, is refuted by the fact that dim light preserves bifurcated entrainment only in the LDLD condition with full photophases. Further supporting this conclusion, a prior study—conducted using animals with free-running, unbifurcated rhythms—found that dim light did not enhance nonparametric phase resetting to bright light pulses (5 min, 100 lux) when full phase response curves (PRCs) were generated with dimly lit versus completely dark background conditions (Evans et al., 2007).

Dim scotophase illumination facilitates the initial induction of bifurcated entrainment under LDLD both by interacting with stronger stimuli after entrainment to photoperiods with short nights (Evans et al., 2005) and changing the coupling between multiple circadian oscillators (Gorman & Elliott, 2004; Gorman et al., 2006). Thus, dim light may facilitate the maintenance of bifurcated entrainment under fLDLD either because dim light (1) moderates the intrinsic tendency of oscillators to rejoin—a coupling subhypothesis; or (2) enhances the efficacy of the bright photophase illumination to counteract this tendency—a bright-light efficacy subhypothesis; or (3) modulates both oscillator coupling and the efficacy of bright light. Consistent with a coupling hypothesis, unbifurcated, free-running rhythms are altered by dim illumination under conditions where effects of bright light are eliminated (i.e., both period and activity duration are longer in constant dim light than constant darkness) (Evans et al., 2007). Analyses of *Tupaia* rhythms likewise point to an effect of dim light on oscillator coupling (Meijer et al., 1990). In contrast, dim light produced only a modest effect on the rapid rejoining of bifurcated rhythms that occurred after transfer from DIM-fLDLD into constant conditions (Evans et al., 2010). Because rejoining in that study occurred very quickly, however, it is not clear that it would have been possible to detect a modest effect on mutual oscillator coupling that may, nonetheless, be functionally important under full or skeleton LDLD cycles. The coupling, but not the efficacy, model, moreover, is consistent with the finding that dim light destabilized bifurcation in Experiment 3 when sLDLD were introduced 5 wks after transfer to LDLD. In that experiment, loss of bifurcation under DIM-sLDLD was often associated with the further dissociation of activity rhythms into more than two components (cf. Figure 2K). Similarly, activity rhythms can be dissociated into more than two components by exposure to LD3:3 and LD5:1, but only when daily scotophases incorporate dim illumination (Evans & Gorman, unpublished observations). Thus far, the evidence suggests that the nature of the dim-light preservation of bifurcation under fLDLD results from a diminished tendency for oscillators to rejoin that is effectively countered by the full 7-h photophases. Under sLDLD cycles, dim-light may exert further dissociating effects that prevent maintenance of cleanly bifurcated rhythmicity when sLDLD cycles are introduced abruptly after only a few weeks of bifurcated entrainment.

Animals in the present studies exhibited a substantial rate of spontaneous rejoining prior to manipulation of the scotophase conditions, especially in animals that initially bifurcated after a cage change. Different dynamics following initial transfer to LDLD versus after a cage change have been described previously (Evans et al., 2005; Gorman et al., 2003). These discrepancies in the dynamics of induction and maintenance of the bifurcated entrainment state may reflect stable individual differences in basic circadian organization or, alternatively, the different entrainment conditions under which rhythm bifurcation is induced (e.g., after LD14:10 versus LDLD7:5:7:5). All three of the present studies had high rates of rejoining in comparison with some other published studies from our laboratory (cf. Gorman & Elliott, 2003; Gorman & Steele, 2006), which consequently allowed detection of both stabilizing and destabilizing actions of DIM light. Other factors that influence bifurcation stability (e.g., genotype, presence of the wheel, scotophase duration, dim- and bright-light intensity) await more systematic investigation.

In summary, dim scotophase illumination enhances the stability of the bifurcated state in fLDLD but not in sLDLD, which indicates that dim scotophase illumination interacts with the parametric effects of bright light during the photophase. Stabilization by DIM of the bifurcated state in fLDLD likely derives from a change in the interactions between oscillators and/or in the ability of bright-light photophases to counteract those interactions. Parametric actions of bright light are critical to prevent oscillator rejoining, but the apparent destabilization by DIM of the bifurcated state in sLDLD implies that dim light has actions beyond interacting with these parametric actions. The most parsimonious explanation, supported by related studies (Evans et al., 2007, 2010; Gorman & Elliott, 2004; Gorman et al., 2006), is that dim light affects oscillator coupling, which in turn governs the waveform of the circadian oscillator and its stability under a range of lighting conditions. It is worth noting that irradiance levels of the dim nighttime illumination used in the present study fall well below intensities traditionally associated with circadian entrainment to LD cycles (but see Butler & Silver, 2010) and induction of significant phase shifts by relatively brief light pulses (Evans et al., 2007). We employed a narrow bandwidth green LED light source, both for historical reasons and for analytical advantages related to quantitation of photobiological mechanisms. Our artificial dim light is comparable in illuminance and irradiance, if not its spectrum, to that experienced in nature under a nighttime sky. Natural lighting of this intensity is known to influence the nocturnal behavior of various species in the field

and under laboratory conditions (Bachleitner et al., 2007; Brainard et al., 1984; Erkert & Grober, 1986; Fernandez-Duque & Erkert, 2006). Ongoing studies that investigate the physiological and genetic mechanisms required for the transmission of dim scotophase illumination should provide critical insight into how the circadian system is affected by dim light at night. A further understanding of these novel actions of dim illumination should provide insights into the potential plasticity of circadian rhythmicity and enhance efforts to manipulate clock function for therapeutic purposes.

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