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Circadian Effects of Light No Brighter Than Moonlight

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Abstract In mammals, light entrains endogenous circadian pacemakers by inducing daily phase shifts via a photoreceptor mechanism recently discovered in retinal ganglion cells. Light that is comparable in intensity to moonlight is generally ineffective at inducing phase shifts or suppressing melatonin secretion, which has prompted the view that circadian photic sensitivity has been titrated so that the central pacemaker is unaffected by natural nighttime illumination. However, the authors have shown in several different entrainment paradigms that completely dark nights are not functionally equivalent to dimly lit nights, even when nighttime illumination is below putative thresholds for the circadian visual system. The present studies extend these findings. Dim illumination is shown here to be neither a strong zeitgeber, consistent with published fluence response curves, nor a potentiator of other zeitgebers. Nevertheless, dim light markedly alters the behavior of the free-running circadian pacemaker. Syrian hamsters were released from entrained conditions into constant darkness or dim narrowband green illumination (~ 0.01 lx, 1.3×10^{-9} W/cm², peak $\lambda = 560$ nm). Relative to complete darkness, constant dim light lengthened the period by ~ 0.3 h and altered the waveform of circadian rhythmicity. Among animals transferred from long day lengths (14 L:10 D) into constant conditions, dim illumination increased the duration of the active phase (α) by ~ 3 h relative to complete darkness. Short day entrainment (8 L:16 D) produced initially long α that increased further under constant dim light but decreased under complete darkness. In contrast, dim light pulses 2 h or longer produced effects on circadian phase and melatonin secretion that were small in magnitude. Furthermore, the amplitude of phase resetting to bright light and nonphotic stimuli was similar against dimly lit and dark backgrounds, indicating that the former does not directly amplify circadian inputs. Dim illumination markedly alters circadian waveform through effects on α , suggesting that dim light influences the coupling between oscillators theorized to program the beginning and end of subjective night. Physiological mechanisms responsible for conveying dim light stimuli to the pacemaker and implications for chronotherapeutics warrant further study.

Key words dim light, circadian visual system, irradiance, constant conditions, phase shift, phase response curve, PRC

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In mammals, a circadian pacemaker within the suprachiasmatic nuclei entrains to the solar day via daily phase shifts induced by light (Pittendrigh and Daan, 1976a). Hallmark circadian responses to light, such as phase resetting and melatonin suppression, are characterized by properties (e.g., photon integration, unique spectral tuning, and high irradiance thresholds) that distinguish them from visual responses mediated by the rod and cone photoreceptors underlying image formation (Brainard et al., 1982; Nelson and Takahashi, 1991a, 1991b). Circadian entrainment to light remains intact in transgenic mice lacking rods and cones but is eliminated with additional ablation of the gene for the photopigment melanopsin that is normally expressed in a subpopulation of retinal ganglion cells (Hattar et al., 2003; Lucas et al., 2001). Because these melanopsin-containing retinal ganglion cells project to the SCN, are intrinsically light sensitive, and display properties similar to circadian photic responses studied in vivo (Berson, 2003; Gooley et al., 2003), they are the leading candidate for the primary photoreceptor mechanism of the circadian system.

Nocturnal rodents, such as the Syrian hamster, navigate in the field under nighttime illumination as high as 0.04 and 0.3 lx at quarter and full moon, respectively (Biberman et al., 1966; Thorington, 1980). Light of this intensity, while enough to see by, has been shown in the lab and the field to be largely ineffective in suppressing melatonin or inducing phase shifts in the hamster (Brainard et al., 1982; Nelson and Takahashi, 1991a, 1991b). Comparative studies of light responses among rodents inhabiting canopied forest versus open deserts have suggested that the sensitivity of the circadian visual system may have been titrated adaptively so that the central pacemaker is unaffected by natural nighttime illumination (Brainard et al., 1984; DeCoursey, 1990; Nelson and Takahashi, 1991b).

These results notwithstanding, we have shown that circadian entrainment in the nocturnal hamster is demonstrably sensitive to dim nighttime illumination (~0.01 lx) provided by narrowband light-emitting diodes (LEDs, peak $\lambda = 560$) under a variety of behavioral paradigms (Gorman et al., 2006). For example, after transfer from long day to short day lengths, Siberian hamsters exposed to dimly lit nights rather than dark nights display accelerated photoperiodic responses (e.g., expansion of nocturnal activity [α], gonadal regression, and weight loss) and reductions in the incidence of short day "nonresponsiveness" (Gorman and Elliott, 2004). Furthermore, in Syrian

hamsters held under non-24 h T-cycles with dimly lit nights, the upper range of entrainment is increased by ~4 h relative to T-cycles with completely dark nights (Gorman et al., 2005), and in both species of hamster, dimly lit nights facilitate bimodal entrainment, or "splitting," under 24-h light:dark:light:dark cycles (Evans et al., 2005; Gorman and Elliott, 2004; Gorman et al., 2003). Thus, completely dark nights are not functionally equivalent to dimly lit nights, even when nighttime illumination is below putative thresholds for the circadian system.

The present studies assess which of 3 nonexclusive hypotheses may account for the potency of dim illumination under entrained conditions. According to the first hypothesis, our dim illumination is an unexpectedly strong zeitgeber, and some feature of this stimulus causes it to be more potent than predicted from published fluence-response curves (e.g., its duration or wavelength). Second, dim light could influence re-entrainment by modulating the magnitude of phase resetting by stronger zeitgebers, such as bright light or nonphotic stimuli. Alternatively, dim light changes a basic property of the circadian pacemaker (e.g., period [τ], α , or amplitude). Here we find that circadian waveform, as reflected in α , is markedly altered under constant dim light relative to complete darkness. We interpret our results as evidence that dim illumination influences the coupling between circadian oscillators whose phase relations determine the composite shape of the daily rhythm.

MATERIALS AND METHODS

Breeding and Initial Husbandry Conditions

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 (14 L:10 D, lights on: 0300 PST, lights off: 1700 PST, photophase: 100-300 lx, scotophase: 0 lx). After weaning, animals were group-housed without running wheels inside polypropylene cages (48 × 27 × 20 cm) in a room where ambient temperature was maintained at 22 ± 2 °C, with food (Purina Rodent Chow #5001, St Louis, MO) and water available ad libitum.

Procedures

Since estimates of τ can be confounded by a history of phase resetting, we first assessed the

effects of constant dim light and complete darkness on free-running rhythms. Manipulations designed to assess phase resetting to dim light, bright light, and nonphotic stimuli followed.

Free-Running Rhythmicity

At 8 weeks of age, male Syrian hamsters ($N = 59$) were transferred to individual cages located within light-tight environmental chambers (9-10 cages/chamber). Each cage contained a 17-cm diameter running wheel with an opaque plastic guard woven through the rungs to prevent injuries associated with running. Photophase light intensity was ~ 100 lx, and scotophases were dimly lit with green LEDs (12V, Product#LH1049-3702, Arcoelectric, Thousand Palms, CA) mounted externally and facing the back wall of each cage. These LEDs emit a peak transmission λ of 560 nm, with a half maximum bandwidth of 23 nm (Ocean Optics PS1000 spectrometer; Dunedin, FL). LEDs were outfitted with neutral density filters to approximate irradiances used in our previously published studies. 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. Average luminance across positions within an environmental chamber was 0.01 ± 0.001 lx, which is equivalent to an irradiance of 1.3×10^{-9} W/cm² and a photon flux of 3.7×10^9 photons/cm²sec. Previously reported facultative effects of this dim illumination on splitting under 24-h light:dark:light:dark cycles (Evans et al., 2005; Gorman and Elliott, 2004; Gorman et al., 2003) were replicated under the present conditions (DIM nights: 8/8 animals split, DARK nights: 2/8 animals split; Fisher's Exact Test $p < 0.005$; data not shown).

Because prior studies indicated that the effects of dim light may depend on photoperiodic history (Evans et al., 2005), animals were pre-entrained to either a long day photoperiod (LD; 14 L:10 D, lights-on: 0300, $n = 29$) or a short day photoperiod (SD; 8 L:16 D, lights-on: 0600, $n = 30$). After 5 weeks under LD and SD, the house lights in each environmental chamber were permanently extinguished at the lights-off transition (zeitgeber time 12, ZT 12). As illustrated in Fig. 1A, scotophase illumination was either extinguished at ZT 12 (DARK; $n = 20$ /photoperiod) or retained (DIM; $n = 9-10$ /photoperiod). Cage changes were scheduled at 2- to 3-week intervals for specific circadian times (see below) and performed

with the aid of a dim red headlamp (exposure < 2 min/animal).

Dim Light Resetting

To assess whether dim light is a strong zeitgeber, each free-running DARK animal was exposed to a 2 h dim light pulse once every 21 days during weeks 6 to 13 of constant conditions (3 pulses/animal). Under the control of an external electronic timer, all LEDs in a given chamber were simultaneously powered for 2 h. Dim light pulses fell over a broad range of circadian phases since animals had been free running for at least 6 weeks. Two of 4 environmental chambers (1 chamber/photoperiod pretreatment) were pulsed with dim light, and the remaining 2 chambers were left unpulsed as controls. Ten to 11 days later, this arrangement was reversed. Data from this light-pulsing protocol were also used to assess whether 2 h of dim illumination can suppress or augment wheel running.

Nonphotic Resetting

To study effects of dim illumination on nonphotic resetting, we calculated phase shifts resulting from cage changes performed under DIM and DARK conditions (cf. Fig. 1D). To minimize effects on free-running rhythms, initial cage changes (CC#1-3) were scheduled exclusively during subjective night. Subjective day data were obtained during CC#4-5.

Bright Light-Induced Resetting

To assess whether dim light potentiates bright light-induced resetting, a photic phase response curve to a 15 min, 100-lx light pulse was collected against a background condition of complete darkness or dim illumination. To avoid producing confounding changes in α and τ , animals were pretreated with dim light for only 1 week. One week was sufficient to observe marked effects of dim illumination in a prior study (Gorman et al., 2003). Seventeen weeks after release from entrainment, LEDs were repowered for 2 of the 4 DARK chambers (DARK/DIM, 1 chamber/photoperiod pretreatment). After 1 week under DIM, hamsters in these and the remaining 2 DARK chambers were given bright light pulses. Hamsters within each chamber were pulsed simultaneously to sample the full circadian cycle, and then they were left undisturbed for 1 week. LEDs were extinguished for 2 weeks, and then this protocol was repeated with the reversed arrangement.

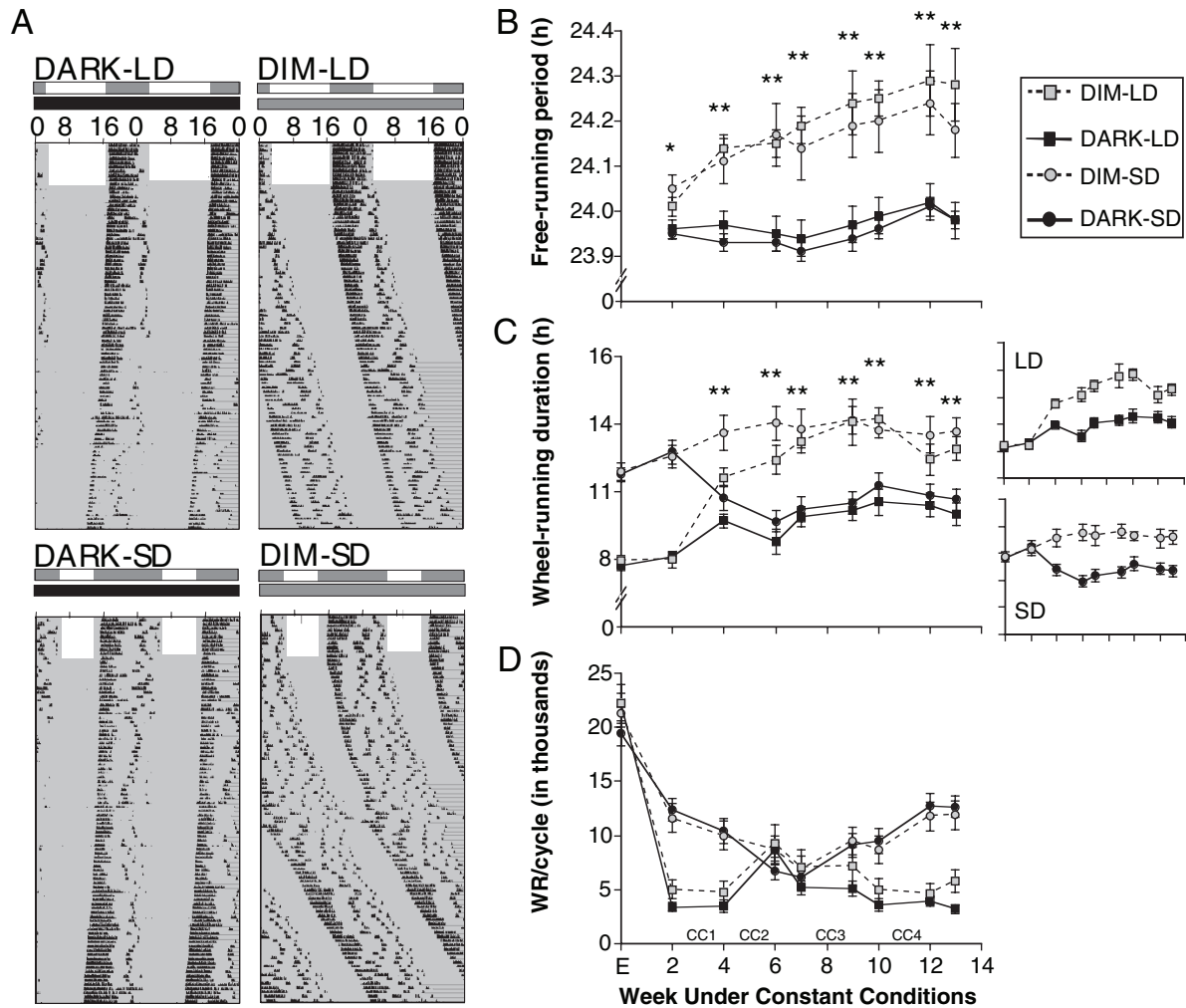


Figure 1. Free-running rhythmicity under constant dim illumination (DIM-) or complete darkness (DARK-). (A) Double-plotted, wheel-running actograms for representative animals entrained to long day (LD) or short day (SD) photoperiods, then released into either DIM or DARK. White and shaded bars above each actogram illustrate the lighting conditions in place during entrainment (top) and after release into constant conditions (bottom). Shading within each actogram depicts the change in lighting conditions. Note that only the last 2 weeks of entrainment are shown. (B-D) Free-running period (τ), activity duration (α), and wheel revolutions per cycle (WR/cycle). τ and α were influenced by scotopic condition (SC) and time after release into constant conditions (Time), whereas WR/cycle was affected by photoperiodic pretreatment (PP) and time. B) τ : SC- $F(1, 45) = 38.8$, Time- $F(8, 38) = 6.5$, SC*Time- $F(8, 36) = 4.2$, $p < 0.001$; PP-, SC*PP-, PP*Time-, SC*PP*Time-, $p > 0.1$. C) α : SC- $F(1, 52) = 35.1$, PP- $F(1, 52) = 11.3$, PP*Time- $F(10, 43) = 11.1$, SC*Time- $F(10, 43) = 8.0$, Time- $F(10, 43) = 5.3$, $p < 0.001$; SC*PP, SC*PP*Time, $p > 0.8$. D) WR/cycle: PP- $F(1, 54) = 22.5$, T- $F(8, 47) = 22.1$, PP*T- $F(8, 47) = 11.3$, $p < 0.0001$; SC*PP*T- $F(8, 47) = 2.7$, $p < 0.05$; SC, SC*PP, SC*T, $p > 0.1$. On the abscissa: E is the last week of entrainment and CC# is the cage change number since release into constant conditions. *DIM-SD versus DARK-SD, $p < 0.005$. **DIM versus DARK for both photoperiodic groups, $p < 0.005$.

Melatonin Suppression

A separate sample of group-housed, female hamsters ($N = 63$, 8 wks of age) was weaned and gradually re-entrained to a reversed 14 L:10 D cycle (lights-on: 2000; photophase illumination: > 100 lx, scotophase illumination: 0 lx). Cage changes occurred once a week during the photophase. After 4 weeks under the reversed 14 L:10 D cycle, the completely dark scotophase (DARK-) was either retained, or LEDs were

powered to provide dim illumination (DIM-) on a single night. Blood samples were collected at 2 points during the scotophase (ZT 17 and ZT 20), and thus, DIM animals received either a 5-h or 8-h dim light pulse (DIM-5h or DIM-8h; $n = 12-16$ /ZT). Three weeks later, a separate group of animals received dim light from ZT 18-20 (DIM-2h; $n = 9$). After 3 more weeks, a subset of DIM- and DARK- animals from the initial blood collection received a 2 h, 100-lx light pulse ($n = 8$ /ZT) as a positive control for melatonin suppression.

Activity Data Collection and Analyses

Half revolutions of home cage wheels triggered closures of a magnetic reed switch, which were recorded and compiled into 6-min bins by VitalView software (Mini-Mitter, Sun River, OR). Actograms were prepared and analyzed using ClockLab software (Actimetrics, Evanston, IL). τ was measured by the slope of a regression line fit to 5 to 7 consecutive activity onsets, excluding the first 4 days after a cage change. Activity onset was defined each day as the first 6-min bin above a threshold value of 15 counts, preceded by at least 1 h of inactivity and followed immediately by 2 consecutive bins above threshold. Activity offset was determined by a similar but opposite rule. α was calculated each day as the difference between activity offset and onset, and the median α for each animal for every week of analysis was recorded. Analyses using mean α yielded results similar to those reported for median α . Total number of wheel revolutions per circadian cycle (WR/cycle) was also quantified for each week of analysis. Further analyses assessing the number of activity bouts per circadian cycle (bouts/cycle) were conducted for weeks 2 and 12 under constant conditions. Distinct bouts were defined as episodes of wheel-running activity surpassing 15 counts, lasting at least 30 min (5 bins) and separated by more than 60 min (10 bins) of subthreshold activity.

Phase shifts in response to dim light, bright light, and nonphotic stimuli were calculated identically. A phase shift was determined for each animal by the displacement between regression lines fit to 5 to 7 consecutive activity onsets before and after the presentation of the stimulus, excluding the first 4 days post-pulse to allow for transient cycles. Circadian time of stimulus presentation was coded as a categorical variable in 4-h bins for quantitative analyses. To assess whether 2-h dim light pulses masked activity levels, total wheel-running revolutions exhibited by each animal during the dim light pulse was subtracted from the total wheel-running revolutions displayed on the previous day at the same clock time. Identical analyses were performed for unpulsed controls using the clock time for pulsed cohorts. Difference scores for pulsed animals and controls were compared across 4-h CT bins.

Melatonin Data Collection

Before blood collection, animals were anesthetized with sodium pentobarbital (55 mg/kg), which was

supplemented with isoflurane. Plasma samples were collected via retinal-orbital bleeds using heparinized caraway micro blood collecting tubes (Fisher, Leicestershire, UK) and transferred to test tubes containing 50 μ L of heparin (1000 units/mL). Melatonin concentration was measured using radioimmunoassay kits, employing an enzymatic pretreatment step (Buhlmann Melatonin Direct RIA, ALPCO, Ltd., Windham, NH). Experimental samples, controls, and standard curve calibrators were incubated with the anti-melatonin antibody and ¹²⁵I-melatonin for 20 h at 2-8 °C. Second antibody was added before a 15-min incubation at 2-8 °C. After centrifugation at 18-28 °C, the unbound supernatant was discarded, and the antibody-bound precipitate was counted via Gamma-counter (Titertek Instruments, Inc., Huntsville, AL). Immunoassay curve-fitting software (Isodata Software; Titertek Instruments, Inc.) used the standard curves to calculate a best-fitting smoothed curve from which sample potency estimates (pg/ml) were obtained algebraically.

Statistics

Most statistical tests were conducted with JMP software (SAS Institute, Cary, NC). Continuously varying circadian measures under free-running conditions were assessed using repeated-measures ANOVA with Bonferroni post hoc tests (Factors: time under constant conditions [Time], scotopic condition [SC], photoperiodic pretreatment [PP], Time*SC, Time*PP, Time*SC*PP). Circadian responses to discrete stimuli presented across the circadian cycle were analyzed using full factorial ANOVA with Bonferroni post hoc tests. ANOVA analyses of phase resetting were supplemented with a recently developed PRC bisection test that permits tests of PRC robustness and between-group comparisons of PRC amplitude (Kripke et al., 2003). Kruskal-Wallis nonparametric tests were used to compare DIM and DARK melatonin levels at each ZT.

RESULTS

Free-Running Rhythmicity

During each week under constant conditions, ~75% of DARK animals exhibited $\tau < 24$ h while ~75% of DIM animals exhibited $\tau > 24$ h. Relative to DARK cohorts, DIM increased τ in SD animals by week 2 and in LD animals by week 4 (Fig. 1B). By

Week 6 under constant conditions, DIM had lengthened τ by ~ 0.2 h relative to DARK. Constant DIM continued to lengthen τ over subsequent weeks, while τ remained essentially unchanged in DARK animals, even after exposure to 2-h dim light pulses during weeks 6 to 13. Photoperiodic pretreatment did not significantly influence τ , nor did it interact with any other factor.

For the first 2 weeks after release from entrainment, SD animals had longer α than LD animals, regardless of whether constant conditions were DIM or DARK (Fig. 1C). Over subsequent weeks, DIM-LD and DARK-LD animals lengthened α by ~ 4 h and ~ 1.5 h, respectively (Fig. 1C, inset top). Unexpectedly, the long α produced by SD was maintained under DIM, but not under DARK conditions (Fig. 1C, inset bottom). DIM-SD animals increased α by ~ 1.5 h from initial free-running values, whereas DARK-SD animals decreased α by ~ 2 h. Consequently, α values corresponded to scotopic condition and not photoperiodic history after 6 weeks under constant DIM and DARK conditions.

Release from entrainment led to a marked decrease in wheel-running amplitude (WR/cycle), which was influenced thereafter by photoperiodic history and not by scotopic condition (Fig. 1D). For the first few weeks after release, WR/cycle was lower for LD groups than SD groups. After ~ 10 weeks under constant conditions, SD animals then increased WR/cycle, likely in response to gonadal recrudescence. LD animals similarly increased WR/cycle ~ 5 weeks later, just prior to bright light manipulations (data not shown).

Bout analyses characterizing the distribution of activity under constant conditions indicate that DIM promotes the fragmentation of the active phase (Fig. 2). At week 12, hamsters under DIM displayed more bouts/cycle than their DARK counterparts, and there were no differences based on photoperiodic history. At week 2, SD animals displayed more bouts/cycle than LD animals, and DIM animals displayed more bouts/cycle than DARK animals. Background illumination thus altered the structure of the active phase before its duration, since the latter did not manifest until week 4 of constant DIM.

Phase Resetting

Exposure to 2-h dim light pulses yielded a statistically robust PRC (Fig. 3A; PRC bisection test, $p < 0.05$), with a 45-min difference between peak advances and delays. Significant phase advances

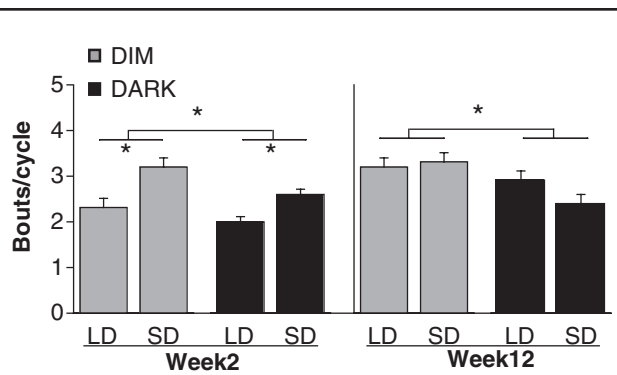


Figure 2. Mean (\pm SEM) number of activity bouts displayed per circadian cycle (bouts/cycle), either 2 or 12 weeks after release from LD or SD into constant conditions. During week 2 of constant conditions, bouts/cycle were greatest in SD and DIM animals (PP: $F[1, 55] = 27.6$, $p < 0.0001$; SC: $F[1, 55] = 7.8$, $p < 0.01$; SC*PP, $p > 0.3$). During week 12, bouts/cycle were greatest in animals under DIM conditions (SC: $F[1, 55] = 10.26$, $p < 0.01$; PP, SC*PP, $p > 0.1$). * $p < 0.01$. DIM = dim illumination; DARK = complete darkness; LD = long day; SD = short day.

were elicited during late subjective night (CT20-24, $p < 0.001$), and the PRC for dim light pulses was similar in shape to that obtained with 15-min bright light pulses (Fig. 3B). Bright light PRCs collected against DIM and DARK conditions were both statistically significant (PRC Bisection test, $p < 0.0001$ in both cases), and background conditions did not significantly alter PRC amplitude or shape (PRC Bisection test, $p > 0.1$; ANOVA: SC, SC*CT, $p > 0.2$).

Under both DIM and DARK background conditions, cage changes produced significant nonphotic PRCs (PRC Bisection test, $p < 0.01$ in both cases) characterized by large phase advances during subjective day (Fig. 3C). PRC amplitude was not influenced by background condition (PRC bisection test, $p > 0.05$), although the shape of the nonphotic PRC was affected (SC*CT: $F[5, 237] = 2.97$, $p < 0.05$). Cage changes conducted during late subjective day elicited significant phase advances from animals in DIM, but not DARK (CT9-12, ANOVA LSM contrasts, $p < 0.009$). Additionally, cage changes conducted during early subjective day tended to produce phase advances from animals in DARK but not DIM (CT1-4, ANOVA LSM contrasts, $p < 0.03$), although this test did not meet the criteria for multiple comparisons.

Behavioral Masking

Relative to unpulsed controls, animals given 2-h dim light pulses did not display a significant

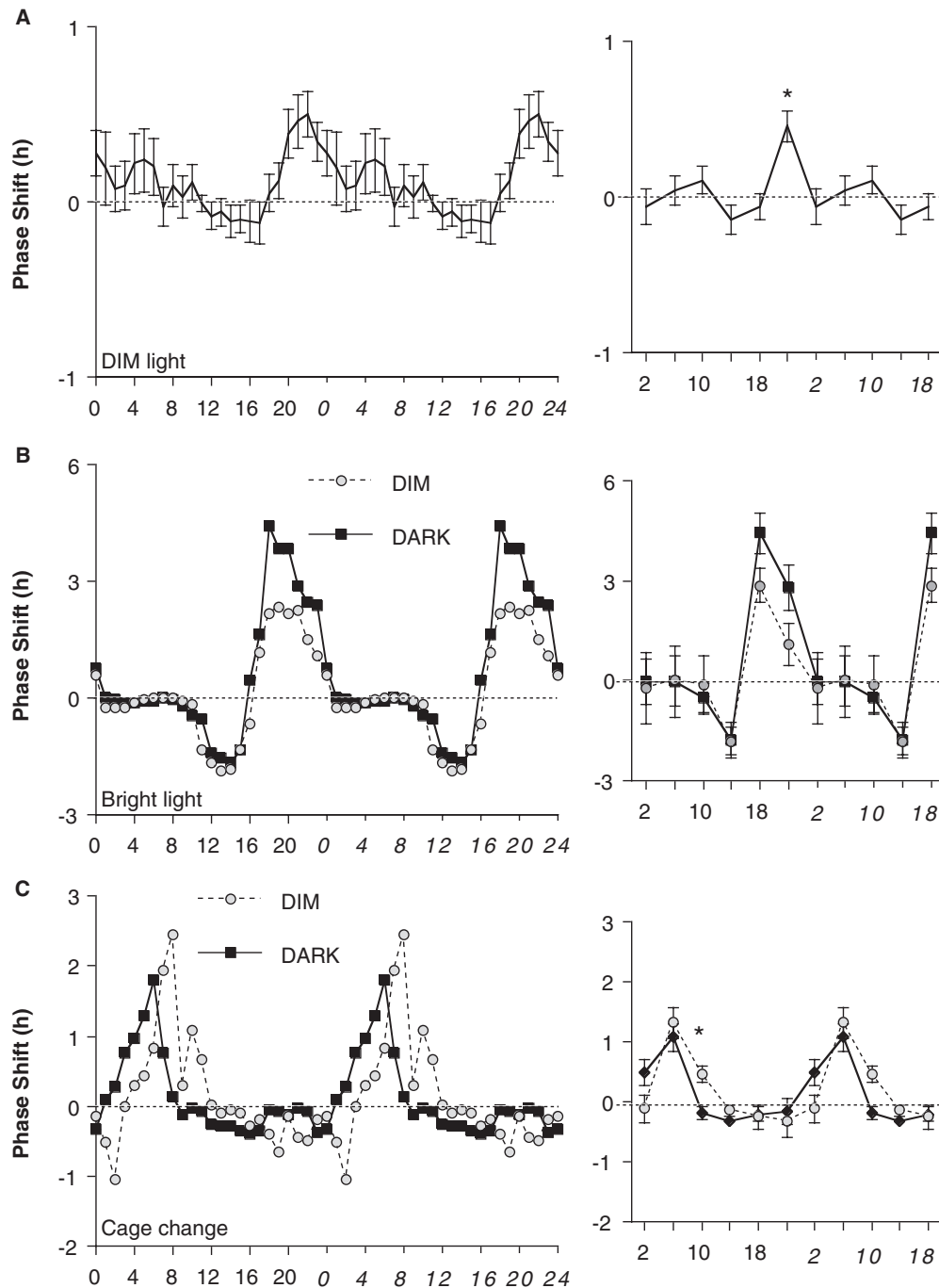


Figure 3. Phase response curves for photic and nonphotic stimuli as drawn with a 3-h moving average (left) or as grouped in 4-h bins for quantitative analyses (right). (A) PRC for 2-h dim light pulses provided against complete darkness. Stippled line at zero represents mean values of controls maintained in darkness. (B) Bright light PRC for 5 min, 100 lx light pulse collected against DARK or DIM background conditions. Prior to bright light pulses, animals were exposed to dim illumination for 1 week. Note the difference in the scale of the y -axis relative to the DIM light PRC. (C) Nonphotic PRC for cage changes performed under constant DIM and DARK conditions once every 2 to 3 weeks. Error bars have been removed from smoothed bright light and nonphotic PRCs for clarity. * $p < 0.009$. DIM = dim illumination; DARK = complete darkness.

change in wheel-running levels relative to the preceding day. This result was obtained when analyses included all data (ANOVA, $p > 0.05$) and when

analyses were restricted to 4-h CT bins during the active phase of the circadian cycle (ANOVA, $p > 0.1$).

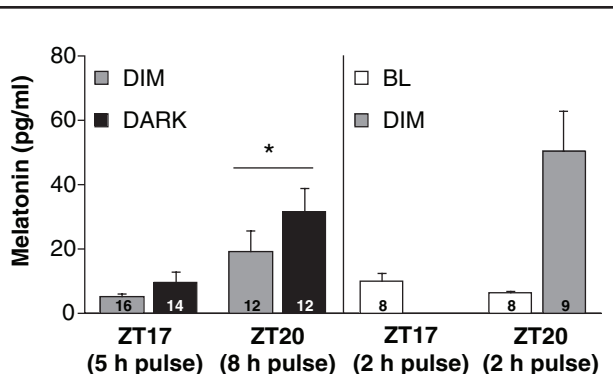


Figure 4. Mean (\pm SEM) serum melatonin concentrations of hamsters sampled under dim or dark conditions at different phases of a 14 L:10 D cycle. DIM light pulses 8 h long, but not 2 h or 5 h long, produced significant melatonin suppression. * $p < 0.05$. DIM = dim illumination; DARK = complete darkness; BL = bright light.

Melatonin Suppression

8-h DIM light pulses significantly suppressed melatonin secretion relative to levels exhibited by DARK controls at ZT 20 (Fig. 4; $\chi^2(1) = 4.6$, $p < 0.05$). At ZT 17, melatonin levels were lower than at ZT 20 and were not significantly different under DIM and DARK conditions ($\chi^2(1) = 1.6$, $p > 0.1$). After 2-h DIM light pulses, melatonin levels showed no evidence of suppression and were not different from DARK controls sampled 3 weeks earlier ($\chi^2(1) = 1.6$, $p > 0.1$). Lastly, 2-h bright pulses delivered at the end of the experiment yielded low melatonin values at ZT 20, which differed from the DARK-ZT 20 animals that were sampled 6 weeks earlier (post hoc comparison; $\chi^2(1) = 13.7$, $p < 0.001$).

DISCUSSION

In the wild, the moon and stars dimly light the nighttime landscape. Under laboratory conditions, dim nighttime illumination of comparable intensity is a potent modulator of circadian entrainment in hamsters held under a variety of behavioral paradigms (Gorman et al., 2006). The present study confirms that such dim light is only a very weak zeitgeber on its own, producing at most a 30-min phase advance with a 2-h pulse. After dim light pulses of more standard duration (e.g., 15 min), the phase shift would be expected to be nondetectable. Furthermore, dimly lit versus completely dark background conditions do not augment the phase-shifting capacities of bright light

or cage changes. Instead, constant dim light and darkness cause the circadian pacemaker to exhibit markedly different free-running properties. As discussed further below, these latter actions provide insight into potential mechanisms underlying the effects of dim illumination under entrained conditions. It remains to be determined how actual moonlight and starlight—with spectral and temporal features not presently simulated—can influence circadian processes in nature. Nevertheless, the use of nighttime illumination may be an attractive and effective means for manipulating the circadian systems of other mammals, including humans. Further characterization of its effects may provide insight into basic clock mechanisms.

Circadian phase resetting exhibits a monotonic dependence on light intensity that is generally well described by a sigmoid-shaped function in a log-linear plot (Nelson and Takahashi, 1991a). Using monochromatic light of 503 nm, Nelson and Takahashi (1991b) estimated the threshold for phase resetting in the nocturnal hamster at 10^{11} photons/cm²sec, a value 27 times greater than the irradiance used here. Accounting for the fact that phase-shifting mechanisms are roughly 8 times more sensitive to photons of ~503 nm than ~560 nm (Takahashi et al., 1984), the effective irradiance of our dim light can be calculated as ~2.3 log units below their estimated threshold. However, the definition of absolute sensitivity can be difficult without large sample sizes that would distinguish between small and null effects. A complementary approach for comparing our stimulus with the published fluence-response curves is to extract, from Naka-Rushton equations, the irradiance predicted to produce phase shifts equivalent to ours (i.e., 30-min phase advances). With 5-min pulses of 500 nm light, an irradiance of 7.5×10^{10} photons/cm²sec is required (Nelson and Takahashi, 1991b). Again accounting for its longer wavelength, our stimulus has an effective irradiance 2.2 log units lower. With perfect temporal summation of photons, the long duration of our dim light pulses (2 h) could account for as much as 1.4 log units of this discrepancy, although previous work demonstrates that photons are less efficiently summated over 1 h than over 5 min (Nelson and Takahashi, 1999, 1991b). The remaining discrepancy may relate to the longer time that animals in the present study were kept in constant conditions before being pulsed with light, since larger phase shifts are commonly observed in this species after prolonged exposure to constant darkness (Pittendrigh et al., 1984; Refinetti, 2006; Shimomura and Menaker, 1994).

Additional methodological differences between present and previous reports (e.g., pulsing procedures and light sources) may also temper absolute comparisons across studies.

Light-induced melatonin suppression is likewise characterized by a sigmoidal relationship between light intensity and response magnitude, but this curve is shifted to lower irradiances than that for phase resetting (Nelson and Takahashi, 1991a). In the present study, melatonin secretion was suppressed nearly 50% by 8 h of dim light, with photon flux approximately one-third that required to elicit half-maximal suppression with a 5-min, 503 nm pulse. Once again accounting for the 8-fold lower efficacy of our longer wavelength stimulus (Takahashi et al., 1984), the effective irradiance is 1.4 log units lower. This discrepancy is reasonably accounted for by our much longer (90-fold) stimulus duration, even given a marked reduction in temporal summation during long light pulses (Nelson and Takahashi, 1999, 1991b). The very low-amplitude PRC and modest melatonin suppression in response to dim light pulses thus afford little direct insight into why animals entrain so differently in dimly lit versus completely dark nights. Indeed, any melatonin suppression accomplished by dim nighttime lighting is insufficient to disrupt the interpretation of the melatonin signal, since dimly lit nights do not compromise gonadal regression in Siberian hamsters held under short day lengths (Gorman and Elliott, 2004).

In previous studies reporting large effects of dim light versus darkness, hamsters were also regularly exposed to bright light and, by necessity, periodic cage changes, raising the possibility that the potency of dim illumination depended on an interaction with these stronger zeitgebers (Evans et al., 2005; Gorman and Elliott, 2004; Gorman et al., 2005). Our complete bright light and cage-changing PRCs reveal that the amplitude of phase resetting to each zeitgeber is not differentially affected by dimly lit and completely dark background conditions (cf. Figs. 3B and 3C), and only the cage-changing curve showed any change in shape. This latter effect may represent a delay in the phase of subjective day rather than an absolute change in the shape of the nonphotic PRC. As demonstrated previously, dim light may interact with cage changes and/or bright light after entrainment to very long day lengths (e.g., 19 L:5 D) but not standard long day lengths (14 L:10 D) (Evans et al., 2005). Likewise, circadian entrainment is not markedly influenced by dimly lit versus completely dark nights under a range of conventional photoperiods (Evans et al., 2005, unpublished observations). Thus, interactions between

dim light and stronger zeitgebers can occur, but these appear restricted to certain conditions and do not provide a general explanation for the effects of dim illumination seen in earlier studies.

In nocturnal rodents, constant light increases τ and decreases α , relationships commonly referred to as Aschoff's first and second rules (Aschoff, 1979, 1960; Pittendrigh, 1960). Consistent with this first "rule," a rule to which Aschoff himself reported variances at low light levels (Aschoff, 1979), constant dim light lengthened τ relative to complete darkness by 0.3 h. As discussed in previous reports, a slight increase in τ by itself is insufficient to account for the ~4 h increase in the upper limit of entrainment (Gorman et al., 2005), enhanced entrainment to short T-cycles (Chiesa et al., 2005), or accelerated short day re-entrainment (Gorman and Elliott, 2004). Contrary to Aschoff's second rule, which is based on fewer studies, constant dim illumination increased α by 3 h, and this proved to be a robust effect maintained over many weeks despite periodic cage changes and dim light pulses. Thus, DIM and DARK conditions produced different steady states, and despite initial differences in α , LD and SD groups converged to common values determined by lighting condition—with α even decreasing in SD animals released into constant darkness.

Previous studies have shown that dim light can increase activity levels (Mrosovsky, 1999), but we found no evidence that the effect of dim light on α was produced by positive masking. First, 2-h pulses and constant dim illumination did not increase wheel revolutions relative to animals held under dark conditions. Furthermore, masking would not account for the gradual increase in α under constant conditions (cf. Fig. 1C), and α did not decrease during the week after the constant dim light was extinguished (unreported data). Lastly, analyses of wheel-running patterns demonstrate that the longer α under dimly lit conditions stems from the redistribution of activity rather than an increase in activity levels. Collectively, these results indicate that masking does not account for the effects of dim light on circadian waveform under entrained or free-running conditions.

This basic change in circadian waveform helps us to understand effects of dim nighttime illumination under entrained conditions. The amplitude of the bright light PRC is proportional to α (Pittendrigh et al., 1984). Therefore, animals held in DIM long enough for α to increase would be expected to have expanded limits of entrainment, as previously reported (Chiesa et al., 2005; Gorman et al., 2005). In this manner, dim light may affect entrainment to

bright light regimens without itself being a strong zeitgeber or direct potentiator of bright light. When dim light is present for only a short period of time (e.g., 1 week as in Fig. 2B), α is not yet altered (DIM: 9.08 ± 0.32 h, DARK: 10.54 ± 0.32 h), and these groups display similarities in the amplitude and shape of the bright light PRC. Empirical studies where α is increased through longer exposure to dim illumination will be critical in assessing whether an increase in the bright light PRC can account for the increase in the upper limit of entrainment in this species. If the effect of dim light on steady-state α can be generalized to other species, this may also explain the accelerated response to short photoperiods in *Phodopus*. This finding, however, does not suggest an immediate explanation for the increased incidence of split rhythms under 24-h light:dark:light:dark cycles (see below).

How does prolonged dim illumination increase α ? The regulation of circadian waveform is best understood in the context of the dual-oscillator model of Pittendrigh and Daan (1976a, 1976b). Briefly, to account for photoperiodic effects on α , these authors posit the existence of "Evening" and "Morning" oscillators with different intrinsic free-running periods and propose that the phase relation between these oscillators determines the length of subjective night. But independent oscillators with different intrinsic periods would be expected to drift in and out of phase with one another over time in constant conditions. Some manner of coupling is therefore needed to account for the fact that a stable long α typically develops in constant darkness and no "criss-crossing" of activity components generally occurs (but see Meijer et al., 1990). If the steady-state α is indeed a reflection of oscillator coupling, then differences in α produced by dim illumination may result from altered oscillator coupling. An effect on circadian coupling might also be inferred from the splitting of activity rhythms under 24-h light:dark:light:dark cycles (Evans et al., 2005; Gorman and Elliott, 2004; Gorman et al., 2003). Indeed, the case for coupling is most strongly made by demonstrating convergent actions of dim light in multiple experimental paradigms for which coupling has been invoked (Gorman et al., 2006). Ongoing work in our laboratory is testing the validity of this approach by addressing whether there is covariation in responses of individual animals to multiple coupling assays (e.g., changes in circadian waveform under constant conditions, short-day photoperiods, and 24-h light:dark:light:dark cycles).

Dim illumination lower than traditionally reported photic thresholds for the circadian visual system has

pronounced effects on circadian waveform and pacemaker function, revealing that the circadian visual system is more sensitive than is perhaps currently appreciated. Other photic stimuli previously assumed to be relatively inconsequential have also been reported to alter free-running and/or entrained circadian rhythms in mammals (Boulos et al., 2002; Erkert et al., 1976; Hofstetter et al., 2005; Kavanau, 1968, 1967; Klante and Steinlechner, 1995; Meijer et al., 1990). Melanopsin-containing photoreceptors possess several unique features that may enable detection of ambient illumination; however, the intrinsic visual responses of melanopsin-containing retinal ganglion cells display high fluence thresholds comparable to those described in vivo (Berson, 2003). Moreover, many photic responses of the SCN (e.g., electrical activity and *c-fos* induction) have similarly high irradiance thresholds (Kornhauser et al., 1990; Meijer et al., 1986; Meijer and Schwartz, 2003), but SCN function can be influenced by rod- and cone-mediated input (Aggelopoulos and Meissl, 2000; Dacey et al., 2005; Dkhissi-Benyahya et al., 2006; Mrosovsky, 2003). Some molecular responses within the SCN are sensitive to lower light levels (Lin et al., 1997), as are visual structures afferent to the SCN (Muscat and Morin, 2006). Ongoing studies assessing the light dependence of the present photic effects on circadian waveform will aid in determining whether they are mediated by physiological mechanisms categorically distinct from those underlying phase shifting and melatonin suppression. Further investigations into the formal and physiological mechanisms underlying the effects of dim illumination may provide novel insights into both the circadian visual system and the circadian pacemaker itself.

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