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Photoperiod alters phase difference between activity onset in vivo and mPer2::luc peak in vitro

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Mickman C, Stubblefield J, Harrington M, Nelson DE. Photoperiod alters phase difference between activity onset in vivo and mPer2::luc peak in vitro. *Am J Physiol Regul Integr Comp Physiol* 295: R1688–R1694, 2008. First published September 3, 2008; doi:10.1152/ajpregu.90510.2008.—Photoperiod is a significant modulator of behavior and physiology for many organisms. In rodents changes in photoperiod are associated with changes in circadian period and photic resetting of circadian pacemakers. Utilizing rhythms of in vivo behavior and in vitro *mPer2::luc* expression, we investigated whether different entrainment photoperiods [light:dark (L:D) 16:8 and L:D 8:16] alter the period or phase relationships between these rhythms and the entraining light cycle in Per2::luc C57BL/6J mice. We also tested whether mPer2::luc rhythms differs in anterior and posterior suprachiasmatic nucleus (SCN) slices. Our results demonstrate that photoperiod significantly changes the timing of the mPer2::luc peak relative to the time of light offset and the activity onset in vivo. In both L:D 8:16 and L:D 16:8 the mPer2::luc peak maintained a more stable phase relationship to activity offset, while altering the phase relationship to activity onset. After the initial cycle in culture, the period, phase, and peaks per cycle were not significantly different for anterior vs. posterior SCN slices taken from animals within one photoperiod. After short-photoperiod treatment, anterior SCN slices showed increased-amplitude Per2::luc waveforms and posterior SCN slices showed shorter-duration peak width. Finally, the SCN tissue in vitro did not demonstrate differences in period attributable to photoperiod pretreatment, indicating that period aftereffects observed in behavioral rhythms after long- and short-day photoperiods are not sustained in Per2::luc rhythms in vitro. The change in phase relationship to activity onset suggests that Per2::luc rhythms in the SCN may track activity offset rather than activity onset. The reduced amplitude rhythms following long-photoperiod treatment may represent a loss of coupling of component oscillators.

circadian rhythm; suprachiasmatic nucleus; mPeriod2; in vitro expression; entrainment

SEASONAL CHANGES IN DAY LENGTH or photoperiod are associated with significant plasticity in behavior and physiology. In mammals, photoperiod changes induce changes in reproductive state, coat color, and thermoregulation, as well as changes in behavior and activity levels (3). Behavioral activity rhythms in mice and hamsters entrained to long- or short-day photoperiods reveal long-lasting changes in the timing of activity onset and activity duration, as well as the period, and photic responsiveness of circadian activity rhythms (15). Coincidence of a light-sensitive circadian clock phase with light exposure during long photoperiods (but not during short photoperiods) may serve as a mechanism for photoperiod detection (5).

In mammals the suprachiasmatic nucleus (SCN) of the hypothalamus functions as a light-responsive circadian pacemaker, maintaining its own endogenous rhythm and synchronizing rhythms in other tissues (20). A complex interaction of up to 15 genes, known as clock genes, and their products drives cellular circadian oscillations (7). Of the known clock genes, the *period1* and *period2* genes are particularly important for circadian responses to environmental light cues. Light increases transcription of mPer1 and mPer2 in the SCN, initiating a cascade that can reset and entrain endogenous circadian rhythms (18).

Exposure to short-day vs. long-day photoperiods changes the timing and expression patterns of *mPer1* and *mPer2* in vivo. *Per2* mRNA remained elevated above baseline in Syrian hamsters for longer durations when housed under long days than when under short days, even after hamsters became refractory to the effects of short days (2, 24). Similar effects are observed for *per1* expression in rats (22), Syrian (10) and Siberian hamsters (9, 15), mice (19), and sheep (8). A long-lasting change in duration and amplitude of clock gene expression may provide a "memory" of photoperiod in the circadian clock system. Such a long-lasting change should be able to be preserved in vitro if an isolated SCN is capable of maintaining this altered gene expression pattern, similar to what has been seen in studies of spontaneous firing rates (e.g., Ref. 12).

Most SCN cells show peak *Per1* expression linked to the time of activity offset under changing photoperiods, although, interestingly, a group of cells in the anterior SCN seems to show a peak linked to activity onset (6). These variations in phase of SCN neurons may lead to changes in the overall circadian waveform that allow the SCN to preserve a code of day length (6, 13, 16, 17, 25).

Here we tested whether in vivo entrainment to long- or short-day photoperiod alters the circadian rhythm of Per2::Luc bioluminescence (27) in the mouse SCN in vitro. First, we determined whether photoperiod pretreatment altered the phase relationship between the light:dark (L:D) cycle, the activity rhythm in vivo, and the phase of Per2::Luc rhythms in vitro. We predicted that the phase of Per2::Luc rhythm in vitro would be influenced by photoperiod and that the Per2::Luc rhythms within many regions of the SCN in vitro would track the time of activity offset, as suggested by prior studies (6, 13). Second, we tested the hypothesis that short and long photoperiods would change Per2::Luc rhythm amplitude and duration in vitro in a manner similar to that measured for *mPer2* expression in vivo. Finally, we tested whether period aftereffects

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observed in behavioral rhythms in vivo after long- and short-day photoperiods (15) would also be observed in Per2::Luc rhythms in vitro.

MATERIALS AND METHODS

Research was performed in accordance with the "Guiding Principles in the Care and Use of Animals" (American Physiological Society), and protocols were reviewed and approved by the Institutional Animal Care and Use Committees of the University of St. Thomas and Smith College, an Association for Assessment and Accreditation of Laboratory Animal Care-certified facility.

mPer2::Luc bioluminescence. Adult male and female C57BL/6J *mPer2^{Luc}* (*mPeriod2^{Luciferase}*) mice were bred from in-house colonies (founders courtesy of Dr. E. Herzog, Washington University, St. Louis, MO; originally derived from the colony of Dr. J. Takahashi, Northwestern University, Evanston, IL). For the initial experiment mice were group housed (4 per cage) for 3–4 wk under L:D 16:8 (16-h light:8-h darkness, lights on at 0400; $n = 13$) or 4–5 wk under L:D 8:16 (lights on at 0800; $n = 12$). In a subsequent trial we tested mice entrained to L:D 18:6 for 4 wk with lights on at 0300 ($n = 9$). For all experiments food and water were available ad libitum. Mice were 9–14 wk of age at the time of slice preparation and were homozygous for the *mPer2^{Luc}* gene.

Mice were killed in the light (at times ranging from 2 to 10 h before lights off) by halothane overdose. The brains were rapidly removed and placed in ice-cold HBSS (Invitrogen, Carlsbad, CA). Coronal sections (300 μm) were made from the anterior and posterior SCN with a vibratome. The majority of the SCN was generally contained within these two sections; therefore, medial SCN is represented in both sections. We compared anterior and posterior SCN sections from the same animal as well as across animals. Hypothalamic sections were trimmed to the SCN and optic chiasm and placed on culture plate inserts (model PICMORG50; Millpore, Billerica, MA) in 35-mm petri dishes (BD Faclon, Franklin Lakes, NJ) with 1.2-ml sterile-filtered (Nalgene, Rochester, NY) culture media [composed of 1×DMEM (cat. no. D5030; Sigma-Aldrich, St. Louis, MO) supplemented with 1×B-27 (Invitrogen), 4 mM L-glutamine (Invitrogen), 25 mM glucose (Sigma), 4.2 mM Na_3PO_4 (Sigma), 10 mM HEPES (Sigma), 25 U/ml penicillin G sodium (Invitrogen), 34 μM streptomycin sulfate (Invitrogen), and 100 μM beetle luciferin (Promega, Madison, WI). Cultures were maintained at 35.8°C, and bioluminescence was recorded for 1-min durations at 10-min intervals over 7 days with a photomultiplier tube and turntable (Lumicycle, Actimetrics, Evanston, IL).

The Lumicycle analysis package was used to analyze the bioluminescence rhythms. *Cycle 0* data (from time of slice preparation through midnight of that evening) were excluded from the phase and period analyses, and only slice records containing three mPer2::luc peaks were used. Records meeting these criteria showed significant rhythms as assessed by χ^2 periodogram. Lumicycle was used to fit a low-order polynomial to each data set to determine the baseline, which was then subtracted from the raw data. Analyses were then performed on the baseline-subtracted rhythms. mPer2::luc peaks were determined by finding the time of maximum amplitude between successive troughs following running-mean smoothing. Using the times of mPer2 peak, linear regression was used to determine period and phase angle difference between the mPer2 rhythms and the entraining L:D cycle on *day 0* for individual slices. To test for an effect of euthanasia time on circadian phase, slices within each photoperiod were assigned to either an early or late time of euthanasia relative to lights off. Within each photoperiod, mice were killed within 6 h of one another. Slices were designated as early if they were prepared within the first 3 h of this range and late if prepared after this duration. Statistical comparisons between times of mPer2::luc peak under different photoperiods were determined with Watson-Williams' *F*-test and Watson's U^2-test for circular data analyses (Oriana, version 2.02c; Kovach Computing). Differences in circadian period were determined with ANOVA and the Tukey-Kramer method (SAS), whereas differences in waveform were determined with ANOVA followed by Bonferroni tests (SPSS). Statistical significance was determined at $P < 0.05$.

Behavioral responses to long- and short-day photoperiods. In a separate experiment, male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 3–4 wk of age and housed under L:D 16:8 or L:D 8:16 ($n = 123$ and 121 per group, respectively) with food and water available ad libitum. After 3 wk mice were transferred to individual cages containing running wheels and microswitches to monitor locomotor activity. Running wheel activity rhythms were recorded and analyzed with a Chronobiology Kit (Stanford Software, Santa Cruz, CA). After preentrainment mice were released into constant darkness (D:D) for 1 wk to allow expression of free-running activity rhythms. These behavioral data were analyzed to determine the circadian period and activity duration (onset and offset) for the rhythm of locomotor activity over the first 7 circadian cycles after L:D 16:8 or L:D 8:16. Activity onsets and offsets were analyzed with eye-fit regression. Phase of activity onset and offset in D:D were extrapolated to the final cycle of L:D to determine the phase relationship between the behavioral rhythms and the entraining light cycle. Statistical differences between activity phases were determined with circular statistics (Oriana, version 2.02c; Kovach Computing) using Watson-Williams' *F*-test and Watson's U^2-test. Statistical differences in circadian period were determined with ANOVA and the Tukey-Kramer method (SAS). Significance was determined at $P < 0.05$.

RESULTS

Comparisons of entrainment phase for rhythms of bioluminescence and behavioral activity. After entrainment to L:D 16:8 or L:D 8:16, rhythms of *mPer2::luc* bioluminescence were obvious for both anterior and posterior SCN slices. The mPer2::luc rhythms from anterior slices were more variable in bioluminescence baseline (Fig. 1) and phase (Figs. 2 and 3) than posterior slices. Rhythms from three randomly chosen animals from each housing condition are shown in Fig. 1.

Significant differences in phase were measured after entrainment to different photoperiods (Figs. 2 and 3). For anterior slices the peak for mPer2::luc rhythms was $+5.88 \pm 2.72$ h (circular mean ± circular SD, mean relative to lights off; $n = 9$) after L:D 16:8 and -0.67 ± 1.63 h ($n = 10$) after L:D 8:16. For posterior slices the peak was $+6.27 \pm 1.08$ h ($n = 10$) after L:D 16:8 and $+0.13 \pm 0.98$ h ($n = 7$) after 8:16. The effect of photoperiod on mPer2::luc phase was significant for both posterior and anterior SCN slices [Watson-Williams *F*-test: $F(1, 15) = 120.0$, $P = 1.5 \times 10^{-8}$; $F(1, 17) = 35.4$, $P = 1.6 \times 10^{-5}$, respectively], but there was no significant phase difference between slices obtained from anterior or posterior SCN within the two photoperiods [Watson-Williams *F*-test: L:D 8:16 slices: $F(1, 15) = 1.299$, $P = 0.271$; L:D: 16:8 slices: $F(1, 17) = 0.164$, $P = 0.60$].

Entrainment to long- or short-day photoperiods also altered running wheel activity rhythms (Fig. 3). Under L:D: 16:8 activity onset was significantly earlier ($+0.87 \pm 0.87$ h) than for mice entrained with L:D 8:16 [-0.13 ± 0.78 h; Watson-Williams *F*-test: $F(1, 242) = 86.1$, $P < 0.001$]. Photoperiod also significantly changed activity offset. After L:D 16:8, offset was significantly earlier (-10.50 ± 2.35 h) compared with activity offset in L:D 8:16 [-15.35 ± 2.18 h; Watson-Williams *F*-test: $F(1, 242) = 270.7$, $P < 0.001$].

Thus photoperiod altered the phase difference between activity onset in vivo and mPer2::luc peak in vitro. This can be

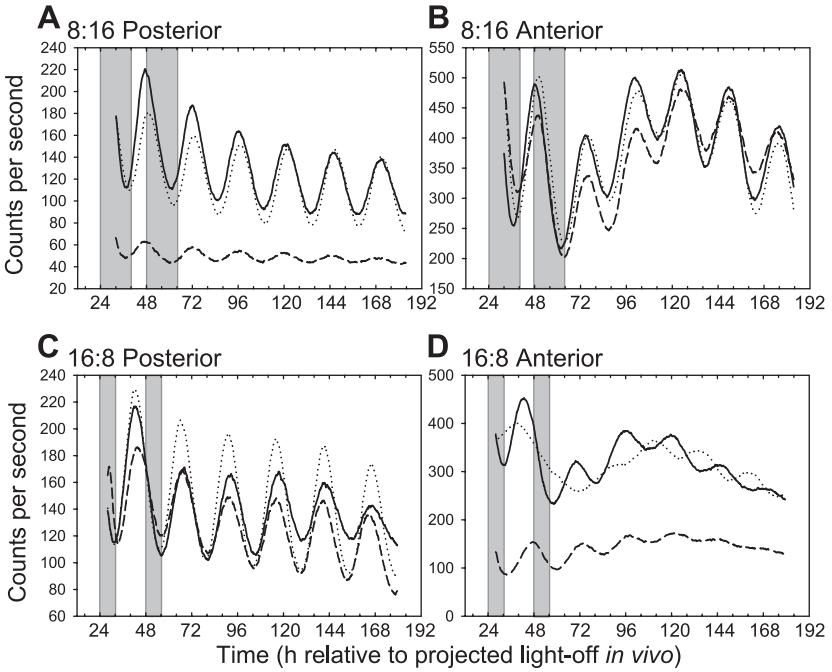

Fig. 1. Representative raw rhythms of Per2::Luc bioluminescence expression in vitro. Three data samples from each photoperiod treatment [*A* and *B* from light:dark (L:D) 8:16; *C* and *D* from L:D 16:8] and location [*A* and *C*, posterior suprachiasmatic nucleus (SCN); *B* and *D*, anterior SCN] are shown; 24 h is the time of onset of darkness in prior L:D. Slice data begin at midnight after slice preparation. Shaded boxes show time of lights off from the prior L:D. Representative animals were chosen randomly. Within each photoperiod, traces with the same line pattern are from the same animal (e.g., solid lines in *A* and *B* represent posterior and anterior slice data, respectively, from the same animal).

seen in Fig. 3. Under long photoperiods, the in vitro Per2::Luc rhythm peaked earlier relative to activity onset than under short photoperiod. The phase of peak Per2::Luc to activity offset was more conserved as photoperiod was altered, while phase relative to activity onset, dark onset, and light onset were all altered after different photoperiods.

We verified that the time of slice preparation did not influence the phase of subsequent mPer2::luc rhythms in vitro. There were no significant differences between the phase of the bioluminescence peak in vitro in early versus late slice preparation times for L:D 8:16 slices [-0.08 ± 1.13 vs. $+0.88 \pm 1.55$ h, respectively, relative to lights off; Watson-Williams *F*-test: $F(1, 16) = 2.083$, $P = 0.168$] or for L:D 16:8 slices [6.35 ± 2.30 vs. 5.27 ± 1.40 h, respectively; Watson-Williams *F*-test: $F(1, 15) = 0.381$, $P = 0.546$]. Within 2–10 h before lights off, differing slice preparation times had no significant effect in *mPer2::luc* phase in vitro.

Comparison of waveform parameters for bioluminescence after L:D 16:8 and L:D 8:16. To determine the influence of photoperiod on mPer2::luc rhythm waveform, we measured peak width (using the times of baseline crossing) and amplitude (mag-

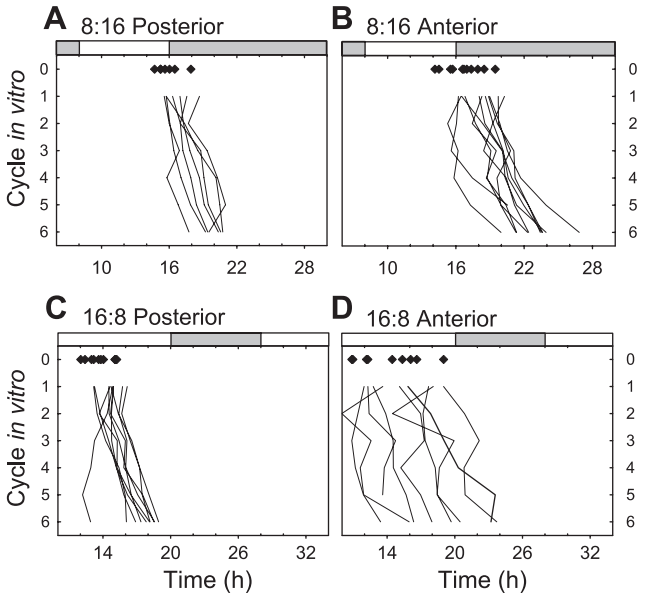

Fig. 2. Times of Per2::Luc peaks over *cycles 1–6* in vitro. Lines connect successive times of Per2::Luc peaks. All data are shown for 2 photoperiod treatments (*A* and *B* from L:D 8:16; *C* and *D* from L:D 16:8) and SCN locations (*A* and *C*, posterior SCN; *B* and *D*, anterior SCN) for *cycles 1–6* in vitro. Light-dark bars show L:D before slice preparation. Data points on *cycle 0* represent extrapolated times of Per2 peak expression on *cycle 0* made from individual rhythms with linear regression.

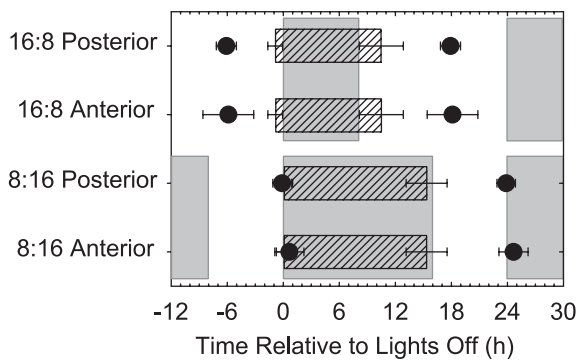

Fig. 3. Phase of in vitro mPer2 expression peak relative to in vivo locomotor activity and entraining L:D. Times of darkness under entraining light cycle are shown by gray shaded boxes for L:D 16:8 and L:D 8:16 (aligned and used as the phase reference point for analyses). Times mPer2 peak on *cycle 0* (●, circular mean $\mu \pm$ circular SD) are double plotted with the corresponding behavioral activity rhythm (horizontal cross-hatched bars, activity onset and offset, circular mean $\mu \pm$ circular SD). Within L:D 16:8 and L:D 8:16 the peak mPer2::luc phases from the anterior SCN were not significantly different from those measured in posterior SCN. Overall (anterior and posterior slices) the phase of the mPer2::luc rhythm after L:D 16:8 was significantly different from the phase after L:D 8:16 (Watson-Williams *F*-test, $P < 0.001$). Phases of both activity onset and offset were also significantly different under L:D 16:8 and L:D 8:16 (Watson-Williams *F*-test, $P < 0.001$).

nitude of peak over mean level) for the *day 1* in vitro data. Peak width showed a significant difference across groups [$F(3, 33) = 4.276$, $P = 0.012$]. Bonferroni post hoc tests indicated that this was due to a smaller peak width in the posterior slices from the short photoperiod compared with the anterior slices in the long photoperiod (mean ± SE: 12.19 ± 0.25 vs. 14.54 ± 0.69 h). The groups also showed differences in amplitude of the waveform [$F(3, 33) = 9.59$, $P < 0.001$], with post hoc tests revealing that the anterior slices from the short photoperiod showed higher-amplitude waveforms than all the other groups, whereas the other three groups did not show statistical differences from each other (see Fig. 4).

In a final experiment we entrained mice to L:D 18:6 [similar to the photoperiod used by Inagaki et al. (6)] and compared the rhythm waveforms with rhythms after L:D 16:8 and L:D 8:16. To better reveal rhythm differences, we did not smooth or detrend the bioluminescence data and we focused our comparisons on only the initial cycles in vitro. Data from all three photoperiods are shown in Fig. 5. For all three photoperiods and for both anterior and posterior slices there appears to be large between-slice variability in mPer2::luc bioluminescence on the initial cycle in vitro following tissue dissection. The larger variability among rhythms was present for 12–24 h in vitro, and then a more consistent rhythmic pattern emerged. This initial variability often includes an initial "spike" in bioluminescence within the first 1–3 h of placing the tissue in culture, followed by a second, usually smaller peak occurring ~3–5 h later. There was no consistent pattern for the timing or presence of the second peak relating to either photoperiod or SCN location. By 24–36 h in culture, most cultures showed a consistent rhythm that was more obviously circadian in nature and more similar in appearance to that observed in subsequent cycles. We saw no indication for double peaks or other obvious differences in rhythm profile after the initial variability.

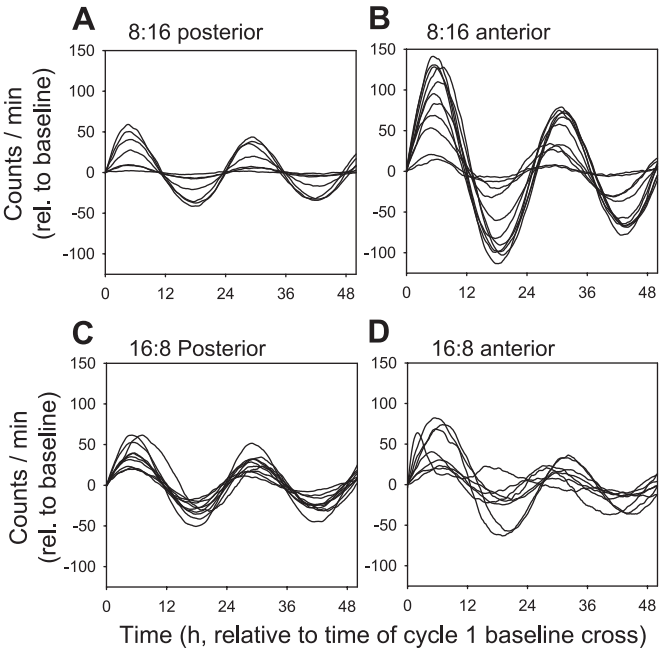

Fig. 4. Waveforms from mice housed under either L:D 16:8 (*C*, *D*) or L:D 8:16. (*A*, *B*) Data have been baseline subtracted and plotted relative to the time of the first half-rise on the first day in vitro. Most, but not all, anterior SCN slices from L:D 8:16 showed increased-amplitude waveforms (see *B*).

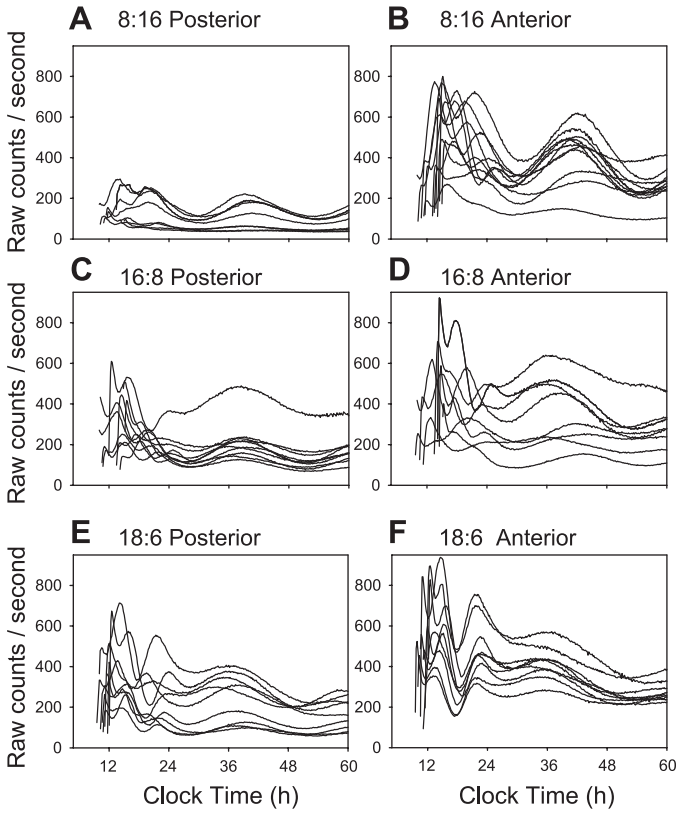

Fig. 5. Raw data for mPer2::luc bioluminescence over initial 3 cycles in vitro after different photoperiods. Data are shown for all slices and entrainment photoperiods (*A* and *B*, L:D 8:16; *C* and *D*, L:D 16:8; *E* and *F*, L:D 18:6). *A*, *C*, and *E* show posterior slice records. *B*, *D*, and *F* show anterior records. Midnight before the day of slice preparation is defined as *cycle time 0*, and data collection for each record begins immediately after slice preparation.

Comparison of period for bioluminescence and behavioral activity. The period of Per2::luc rhythms in SCN slices did not vary with entrainment photoperiod or with location within the SCN. The mean period for anterior and posterior slices from L:D 16:8 was 24.60 ± 0.13 and 24.54 ± 0.13 h, respectively. The periods after L:D 8:16 were 24.89 ± 0.11 and 24.63 ± 0.09 h for anterior and posterior slices, respectively (Fig. 6). There was no significant effect of photoperiod or slice origin on the period of mPer2::luc expression in vitro [photoperiod effect: $F(1, 34) = 2.4$, $P = 0.131$; slice origin effect: $F(1, 34) = 1.79$, $P = 0.190$].

For behavioral rhythms there was a significant effect of photoperiod on the period of activity onset. Animals from L:D 8:16 showed a longer period than those from L:D 16:8 [23.91 ± 0.03 h ($n = 10$) vs. 23.71 ± 0.04 h ($n = 9$); $F(1, 17) = 14.87$, $P = 0.0013$]. The difference in average period in behavioral experiments versus in vitro experiments either may be attributed to the different mouse colonies used in the experiments or may be due to the in vivo/in vitro difference; however, the lack of an effect of photoperiod on period in vitro cannot be attributed to strain differences.

DISCUSSION

Our study demonstrated that photoperiod pretreatment altered SCN phase relative to activity onset and also altered several other rhythm parameters as measured in vitro with Per2::Luc expression. First, we demonstrated that photoperiod pretreatment altered

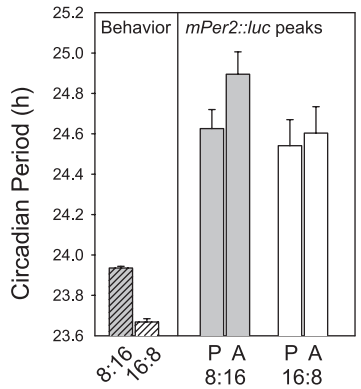

Fig. 6. Circadian period of mPer2 peak expression and corresponding circadian period of behavioral activity rhythm after L:D 16:8 and L:D 8:16. Period of locomotor activity onset is shown for L:D 8:16 (gray hatched bar) and L:D 16:8 (open hatched bar) photoperiods. Period of locomotor activity is significantly different after the 2 photoperiods (ANOVA, $P < 0.05$). Period of Per2 peak expression is shown for posterior (P) and anterior (A) SCN for L:D 8:16 (gray bars) and L:D 16:8 (open bars). There was no significant effect of location within the SCN (anterior/posterior) or of prior photoperiod (L:D 8:16 or L:D 16:8) on period in vitro.

the phase relationship between the Per2::Luc rhythms in vitro and the activity rhythm, changing the phase relationship with activity onset and with the L:D cycle but conserving the phase relationship with activity offset. We did not find evidence for a cell population in anterior SCN linked to time of activity onset, or evidence for a third population of SCN cells, as was observed in a similar study using a *per1* reporter (6, 13). Second, we found that after short-photoperiod treatment anterior SCN slices showed increased amplitude Per2::Luc waveforms and posterior SCN slices showed shorter-duration peak width. Finally, the SCN tissue in vitro did not demonstrate differences in period attributable to pretreatment, indicating that period aftereffects observed in behavioral rhythms after long- and short-day photoperiods are not sustained in Per2::Luc rhythms in vitro. Thus our study brings new questions about how photoperiod induces plasticity in SCN organization.

Whereas in vivo studies indicate many changes in SCN properties in animals housed under varied photoperiods, it is difficult to determine which of these properties are central to changes within the SCN and which are responses of an essentially unchanged SCN to changes in input pathways or to changes in interactions among multiple components of the circadian system. We used the in vitro SCN slice to characterize photoperiod-driven changes in Per2 expression that can be maintained in the isolated SCN. We found that the phase of the Per2 rhythm was shifted in such a way as if to maintain consistent phase with activity offset. It should be noted that changes in duration of activity as photoperiod changes are such that the time of activity offset does not always track light offset. A similar change in phase was also seen in the in vivo measures of *Per1* mRNA (23) and in many in vitro recordings of *mPer1-luc* (6, 13), although some regions and/or cells in the SCN show *mPer1-luc* rhythms phased to activity onset. We hypothesize that the phase of the Per2::Luc rhythm reflects the phase of an underlying oscillation with a strong phase coupling to activity offset.

Electrophysiological, molecular, and simulation studies all suggest that photoperiod responses are coded by changes in phase relation, not waveform, of individual cells (13, 16, 17, 25). We observed evidence for this change in phase relation reflected in the population rhythm as a change in waveform. We revealed an increase in amplitude of the Per2::Luc rhythm specifically in the anterior SCN slices from mice housed under short photoperiod. This increase in amplitude might indicate a tighter coupling of component oscillators, leading to a greater-amplitude population rhythm, or perhaps increased-amplitude cycles within individual neurons. Single-cell imaging experiments would be able to determine which of these explanations might be correct; one such study suggests that individual neurons do not show differences in amplitude of Per1-luc rhythms after different photoperiods but do show greater distribution of phase (13). Simulations suggest that very large changes in single-cell duration would be required for duration changes at the population level (16).

Waveforms were also analyzed for the period of time between the half-rise and the half-fall of the rhythm to search for predicted changes in duration in different photoperiods. Behavioral alpha measurements showed a difference of 4–5 h in activity length, while photoperiodic differences in bioluminescence showed a peak width difference of ~2 h, and this difference was observed only between two groups, with a smaller peak width in the posterior slices from the short photoperiod compared with the anterior slices in the long photoperiod. This provides some support to indicate that the Per2 measure reflects broadened peak duration as observed with electrophysiological measurements and measurements of *per1* in vivo and in vitro, as well as *per2* mRNA measurements in vivo. In mice, photoperiod effects on *per2* SCN mRNA measured in vivo are dramatic, with changes in both amplitude and duration (19). Effects of photoperiod on *per1-luc* waveform duration dissipate over several cycles in vitro (13).

Behavioral studies have demonstrated decreased-amplitude phase shift responses in mice from long photoperiods, an effect seen particularly in cycles with 14 or 16 h of light (21). This effect might arise from effective loss of subpopulations of *per*-expressing neurons from the larger coupled population of cells. This speculative hypothesis assumes that the population of *per*-expressing neurons that stay coupled to each other subserve the photic resetting, and that the magnitude of the phase shift induced by light is proportionate to the number of cells effectively coupled within this population. Our results support this hypothesis in that we observed decreased-amplitude Per2::Luc rhythms after long photoperiods with 16 h of light. We tentatively consider the measurements from Per2::Luc rhythms to be reflective of the summed activity of the coupled cells in the underlying oscillator, but future studies using other markers would be necessary to determine whether this is a valid interpretation. We interpret the decreased-amplitude Per2::Luc rhythm as resulting from loss of coupling of a subset of the population of cells, leaving fewer cells within the coupled group. The alternate interpretation of the decreased-amplitude Per2::Luc rhythm as resulting from decreased amplitude of each individual oscillator would predict increased phase shift amplitude (26), but this is not supported by available behavioral data (21). Our interpretation considers the altered phase-response curve as a network property and suggests that if we could measure the phase-response curve at a single-cell level it would not show changes due to photoperiod pretreatment.

Anterior sections of the SCN were more variable in expression, as can be seen in both representative data records and

summary figures (Figs. 1–3). A similar increase in variability in anterior SCN sections with *Per1-luc* recordings in long photoperiods might be attributed to separate oscillators in the anterior SCN or flexibility in phase relationships and coupling among regions of the SCN (6, 13). It would be interesting to observe the response of these heterogeneous populations when the system is challenged with a light stimulus. We did not observe two peaks in Per2::Luc expression in rostral SCN after long-photoperiod pretreatment, as was reported by Inagaki et al. (6). Interestingly, they observed that after several days in vitro the bimodal peaks merged into a single peak, so it is possible that the Per2::Luc rhythm fuses more quickly than the *per1-luc* rhythms and that explains why we did not observe two peaks. It is also possible that our method for dissection and cutting coronal slices was not sufficient to reveal a parallel subpopulation of Per2::Luc cells, and future studies might better demonstrate this. We did see complex waveforms on the day of dissection, but this was unrelated to SCN region or photoperiod pretreatment and was not sustained into the first full cycle in vitro.

Period aftereffects (changes in free-running period induced after entrainment to long- or short-period light cycles) appear to be important for stabilizing entrainment (15). Using entrainment to T cycles (non-24-h period LD cycles), several studies have demonstrated that the SCN shows paradoxical period aftereffects, in that the SCN period in vitro is negatively correlated with the behavioral rhythm period (1, 11). In our hands, photoperiod pretreatment caused an aftereffect on behavioral rhythm period but had no effect on period of the SCN in vitro. Constant light can lengthen the period of SCN in vitro as well as the behavioral rhythm in vivo (4). Studies looking for evidence of period aftereffects in the isolated SCN give contradictory findings, and further experiments will be needed to clarify the reasons for these disparate findings.

Perspectives and Significance

These findings suggest that a robust outcome of exposure to varied photoperiod is the change in phase of the bulk of Per2-expressing cells relative to activity onset. The change in phase is such that it allows conservation of phase to activity offset, a finding mirrored in recent studies measuring *mPer1* expression in vitro (6, 13). We suggest that this change in phase and the loss of coupling of a subpopulation of the light-responsive cells expressing circadian clock genes might help to explain alterations in photic sensitivity of circadian rhythms with seasonal changes. Changes in internal phase relationships among SCN cells might explain changes in magnitude of photic resetting responses.

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REFERENCES

1. **Aton SJ, Block GD, Hajime T, Yamzaki S, Herzog ED.** Plasticity of circadian behavior and the suprachiasmatic nucleus following exposure to non-24-hour light cycles. *J Biol Rhythms* 19: 198–207, 2004.
2. **Carr AJF, Johnston JD, Semikhodskii AG, Nolan T, Cagampang FRA, Stirland JA, Loudon ASI.** Photoperiod differentially regulates circadian oscillators in central and peripheral tissues of the Syrian hamster. *Curr Biol* 13: 1543–1548, 2003.
3. **Goldman B.** Mammalian photoperiodic system: formal properties and neuroendocrine mechanisms of photoperiodic time measurement. *J Biol Rhythms* 16: 283–301, 2001.
4. **Granados-Fuentes D, Saxena MT, Prolo LM, Aton SJ, Herzog ED.** Olfactory bulb neurons express functional, entrainable circadian rhythms. *Eur J Neurosci* 19: 898–906, 2004.
5. **Hazelrigg DG, Wagner GC.** Seasonal photoperiodism in vertebrates: from coincidence to amplitude. *Trends Endocrinol Metab* 17: 83–91, 2006.
6. **Inagaki N, Honma S, Ono D, Tanahashi Y, Honma K.** Separate oscillating cell groups in mouse suprachiasmatic nucleus couple photoperiodically to the onset and end of daily activity. *Proc Natl Acad Sci USA* 104: 7664–7669, 2007.
7. **Ko CH, Takahashi JS.** Molecular components of the mammalian circadian clock. *Hum Mol Genet* 15, *Spec No* 2: R217–7, 2006.
8. **Lincoln G, Messager S, Andersson H, Hazlerigg D.** Temporal expression of seven clock genes in the suprachiasmatic nucleus and the pars tuberalis of the sheep: evidence for an internal coincidence timer. *Proc Natl Acad Sci USA* 99: 13890–13895, 2002.
9. **Messager S, Hazlerigg DG, Mercer JG, Morgan PJ.** Photoperiod differentially regulates the expression of *Per1* and ICER in the pars tuberalis and the suprachiasmatic nucleus of the Siberian hamster. *Eur J Neurosci* 12: 2865–2870, 2000.
10. **Messager S, Ross AW, Barrett P, Morgan PJ.** Decoding photoperiodic time through *Per1* and ICER gene amplitude. *Proc Natl Acad Sci USA* 96: 9938–9943, 1999.
11. **Molyneux PC, Dahlgren MK, Harrington ME.** Circadian entrainment aftereffects in suprachiasmatic nuclei and peripheral tissues in vitro. *Brain Res.* 1228: 127–134, 2008.
12. **Mrugala M, Zlomanczuk P, Jagota A, Schwartz WJ.** Rhythmic multiunit neural activity in slices of hamster suprachiasmatic nucleus reflect prior photoperiod. *Am J Physiol Regul Integr Comp Physiol* 278: R987–R994, 2000.
13. **Naito E, Watanabe T, Tei H, Yoshimura T, Ebihara S.** Reorganization of the suprachiasmatic nucleus coding for day length. *J Biol Rhythms* 23: 140–149, 2008.
14. **Nuesslein-Hildesheim B, O'Brien JA, Ebling FJP, Maywood ES, Hastings MH.** The circadian cycle of mPER clock gene products in the suprachiasmatic nucleus of the Siberian hamster encodes both daily and seasonal time. *Eur J Neurosci* 12: 2856–2864, 2000.
15. **Pittendrigh CS, Daan S.** A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: pacemaker as clock. *J Comp Physiol* 106: 291–331, 1976.
16. **Rohling J, Meijer JH, VanderLeest HT, Admiraal J.** Phase differences between SCN neurons and their role in photoperiodic encoding; a simulation of ensemble patterns using recorded single unit electrical activity patterns. *J Physiol Paris* 100: 261–270, 2006.
17. **Schaap J, Albus H, vanderLeest HT, Eilers PHC, Detari L, Meijer JH.** Heterogeneity of rhythmic suprachiasmatic nucleus neurons: implications for circadian waveform and photoperiodic encoding. *Proc Natl Acad Sci USA* 100: 15994–15999, 2003.
18. **Shearman L, Zylka M, Weaver D, Kolakowski L, Reppert S.** Two *period* homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. *Neuron* 19: 1261–1269, 1997.
19. **Steinlechner S, Jacobmeier B, Scherbarth F, Dernbach H, Kruse F, Albrecht U.** Robust circadian rhythmicity of *Per1* and *Per2* mutant mice in constant light, and dynamics of *Per1* and *Per2* gene expression under long and short photoperiods. *J Biol Rhythms* 17: 202–209, 2002.
20. **Stratmann M, Schilber U.** Properties, entrainment, and physiological functions of mammalian peripheral oscillators. *J Biol Rhythms* 21: 494–506, 2006.
21. **Stubblefield JS, Nelson DE, Ijichi K.** Entrainment photoperiod modulates the mouse circadian system and its responsiveness to light (Abstract). *Soc Neurosci Abstr* 156.11, 2006.
22. **Sumová A, Jác M, Sládek M, Sauman I, Illnerová H.** Clock gene daily profiles and their phase relationship in the rat suprachiasmatic nucleus are affected by photoperiod. *J Biol Rhythms* 18: 134–144, 2003.

23. **Sumová A, Kováciková Z, Illnerová H.** Dynamics of the adjustment of clock gene expression in the rat suprachiasmatic nucleus to an asymmetrical change from a long to a short photoperiod. *J Biol Rhythms* 22: 259–67, 2007.
24. **Tournier BB, Menet JS, Dardente H, Poirel VJ, Malan A, Masson-Pévet M, Pévet P, Vuillez P.** Photoperiod differentially regulates clock genes' expression in the suprachiasmatic nucleus of Syrian hamster. *Neuroscience* 118: 317–322, 2003.
25. **VanderLeest HT, Houben T, Michel S, Deboer T, Albus H, Vansteensel MJ, Block GD, Meijer JH.** Seasonal encoding by the circadian pacemaker of the SCN. *Curr Biol* 17: 468–473, 2007.
26. **Vitaterna MH, Ko CH, Chang AM, Buhr ED, Fruechte EM, Schook A, Antoch MP, Turek FW, Takahashi JS.** The mouse Clock mutation reduces circadian pacemaker amplitude and enhances efficacy of resetting stimuli and phase-response curve amplitude. *Proc Natl Acad Sci USA* 103: 9327–9332, 2006.
27. **Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, Siepka SM, Hong HK, Oh WJ, Yoo OJ, Menaker M, Takahashi JS.** PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci USA* 101: 5339–5346, 2004.