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

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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 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 differ 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.

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::luc 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^{2 Luc} (mPer2Luc) 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; 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 PICMOR50; Millipore, Billerica, MA) in 35-mm petri dishes (BD Falcon, 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×B27 (Invitrogen), 4 mM L-glutamine (Invitrogen), 25 mM glucose (Sigma), 4.2 mM Na3PO4 (Sigma), 10 mM HEPES (Sigma), 25 μM 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 baseline (Fig. 1) and phase (Figs. 2 and 3) were recorded on a DiB probe (BioLuminescence System, Boston, MA). To ensure that the mPer2Luc gene was expressed and functional, mPer2::Luc rhythms were used. Records meeting these criteria showed significant bioluminescence and behavioral activity.

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.

Comparisons of photoperiod and Per2::Luc rhythms. The photoperiod and Per2::Luc rhythms were compared to the circadian rhythms observed in the anterior region of the SCN (Figs. 2 and 3). For anterior slices the peak for mPer2::Luc rhythms was +5.88 ± 2.72 h (Fig. 2) after L:D 16:8 and +0.67 ± 1.63 h (Fig. 3) after L:D 8:16. For posterior slices the peak was +6.27 ± 1.08 h (Fig. 2) after L:D 16:8 with +0.13 ± 0.98 h (Fig. 3) after L:D 8:16. The effect of photoperiod on mPer2::Luc phase was significant for both anterior and posterior SCN slices [Watson-Williams F-test: F(1, 15) = 120.0, P = 1.5 × 10^{-6}; F(1, 17) = 35.4, P = 1.6 × 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- and short-day photoperiods also altered running wheel activity rhythms (Fig. 3). Under L:D 16:8 activity onset was significantly earlier (+0.87 ± 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
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 \( \text{F}(1, 16) = 2.083, \text{P} = 0.168 \) or for L:D 16:8 slices \( \text{F}(1, 15) = 0.381, \text{P} = 0.546 \). 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. 1. Representative raw rhythms of Per2::Luc bioluminescence expression in vitro. Three data samples from each photoperiod treatment \([A \text{ and } B \text{ from light:dark (L:D) 8:16; } C \text{ and } D \text{ from L:D 16:8}]\) and location \([A \text{ and } C, \text{ posterior suprachiasmatic nucleus (SCN); } B \text{ and } D, \text{ anterior SCN}]\) 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).

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 \((A \text{ and } B \text{ from L:D 8:16; } C \text{ and } D \text{ from L:D 16:8})\) and SCN locations \((A \text{ and } C, \text{ posterior SCN; } B \text{ and } D, \text{ anterior SCN})\) 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::luc 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 \(\mu \pm \text{circular SD}\)) are double plotted with the corresponding behavioral activity rhythm (horizontal cross-hatched bars, activity onset and offset, circular mean \(\mu \pm \text{circular SD}\)). 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, \(P < 0.001\)).
ntitude 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.

**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...
suggest that photoperiod responses are coded by changes in phase of an underlying oscillation with a strong phase coupling to activity offset. We hypothesize that the phase of the Per2::Luc rhythm reflects the time of activity offset. A similar change in phase was also seen in the in vivo measures of activity offset. Changes in photoperiod-driven changes in Per2::Luc rhythms to be reflective of the phase relationship between the Per2::Luc rhythms in vitro and the activity rhythm, changing the phase relationship with activity offset 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 anterior SCN slices showed shorter-duration peak width. Finally, we found that the sum of the coupled cells in the underlying oscillator, measuring neurons from the larger coupled population of cells. This speculative hypothesis assumes that the population of expressing neurons that stay coupled to each other should determine whether this is a valid interpretation. We interpret the results to suggest increased 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 expressing neurons from the larger coupled population of cells. This speculative hypothesis assumes that the population of expressing neurons that stay coupled to each other should determine whether this is a valid interpretation. We interpret the results to suggest increased 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 expressing neurons from the larger coupled population of cells. This speculative hypothesis assumes that the population of expressing neurons that stay coupled to each other should determine whether this is a valid interpretation.
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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