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Distinct patterns of *Period* gene expression in the suprachiasmatic nucleus underlie circadian clock photoentrainment by advances or delays

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Edited by Michael Rosbash, Howard Hughes Medical Institute, Waltham, MA, and approved September 9, 2011 (received for review May 19, 2011)

The circadian clock in the mammalian hypothalamic suprachiasmatic nucleus (SCN) is entrained by the ambient light/dark cycle, which differentially acts to cause the clock to advance or delay. Light-induced changes in the rhythmic expression of SCN clock genes are believed to be a critical step in this process, but how the two entrainment modalities—advances vs. delays—engage the molecular clockwork remains incompletely understood. We investigated molecular substrates of photic entrainment of the clock in the SCN by stably entraining hamsters to T cycles (non–24-h light/dark cycles) consisting of a single 1-h light pulse repeated as either a short (23.33-h) or a long (24.67-h) cycle; under these conditions, the light pulse of the short cycle acts as “dawn,” whereas that of the long cycle acts as “dusk.” Analyses of the expression of the photoinducible and rhythmic clock genes *Period 1* and 2 (*Per1* and *Per2*) in the SCN revealed fundamental differences under these two entrainment modes. Light at dawn advanced the clock, advancing the onset of the *Per1* mRNA rhythm and acutely increasing mRNA transcription, whereas light at dusk delayed the clock, delaying the offset of the *Per2* mRNA rhythm and tonically increasing mRNA stability. The results suggest that the underlying molecular mechanisms of circadian entrainment differ with morning (advancing) or evening (delaying) light exposure, and such differences may reflect how entrainment takes place in nocturnal animals under natural conditions.

A fundamental feature of the circadian clock that regulates daily behavioral and physiological rhythmicity is its entrainment to the environmental cycle of light and darkness. By matching the clock’s endogenous (“free-running”) period to that of the ambient day/night cycle, entrainment ensures that the phase relationship between clock and external day remains stable over time, thereby synchronizing body rhythms to local time. Light is the clock’s preeminent entraining cue (*Zeitgeber*), and the clock responds to light by changing its angular velocity and shifting its phase. To match its oscillation to that of the *Zeitgeber*, the clock can increase its velocity and advance its phase if the clock’s free-running period is too long, or it can decrease its velocity and delay its phase if its free-running period is too short (1, 2).

In mammals, the master circadian clock is located in the suprachiasmatic nucleus (SCN) in the anterior hypothalamus (3). The SCN is composed of multiple, coupled single-cell circadian oscillators (4), and analyses of induced and spontaneous mutations, gene sequence homologies, and protein-protein interactions have identified candidate regulatory molecules and biochemical processes that are likely to constitute the basic intracellular oscillatory mechanism (5). Genes at the core of the clock function within autoregulatory feedback loops, with proteins rhythmically suppressing the transcription of their own mRNAs. When the basic helix–loop–helix transcription factors CLOCK (or NPAS2) and BMAL1 dimerize and bind to E-box enhancer elements, the transcription of the *Period* (*Per1*, *Per2*, and *Per3*) genes is activated. PER proteins accumulate in the cytoplasm, are regulated through their phosphorylation and their association with proteins encoded by the *Cryptochrome* (*CRY1* and *CRY2*) genes, and translocate to the nucleus to negatively regulate their own transcription by interfering with the activity of the CLOCK:BMAL1 dimers. PER2 and Rev-erb-a proteins are part of a second feedback loop that regulates rhythmic *Bmal1* gene transcription. Photic entrainment of the clock must impact the activities or levels of the molecular components of these loops, and the induction of *Per1* and *Per2* gene expression is believed to be a critical step in this process (for references and discussion, see ref. 6), but the precise mechanisms underlying shifts of the phase of the clock or changes in its velocity are not certain.

Here we report our analysis of molecular substrates of entrainment in the SCN of animals photically entrained to T cycles, i.e., non–24-h *Zeitgeber* cycles. We have used a previously described paradigm (7, 8) in which circadian locomotor (wheel running) activity of Syrian hamsters can be stably entrained by a single 1-h light pulse repeated as a T cycle that is either shorter or longer than 24 h by 40 min per cycle. As shown in Fig. 1, the light pulse in the short (23.33-h) or long (24.67-h) cycle falls at a different phase of the clock’s oscillation to attain steady-state entrainment, specifically as a phase-advancing/period-accelerating pulse acting as the short cycle’s “dawn” (preceding the locomotor bout for the nocturnally active hamster) or as a phase-delaying/period-decelerating pulse acting as the long cycle’s “dusk” (preceding the locomotor bout). To determine how differential entrainment by a morning or an evening light pulse affects SCN clock genes, we analyzed the expression of the rhythmic, photoinducible genes *Per1* and *Per2* in groups of hamsters killed at different phases of these cycles. Our results indicate that *Per* gene expression patterns differ under entrainment to short or long T cycles, and suggest that distinct molecular mechanisms underlie these contrasting entrainment modes.

**Results**

**Differential Phase of Entrainment to Short and Long T Cycles.** The different patterns of entrainment to short and long T cycles shown in Fig. 1 are predicted by entrainment theory and previously confirmed in several species, including Syrian hamsters (7, 9, 10). To collect and compare data from groups of animals entrained to these two schedules, we converted time (in hours) to degrees of arc, where one circadian cycle equals 360° (thus, 1° is...
~0.0648 h for animals in the short 23.33-h T cycle and 0.0685 h for animals in the long 24.67-h T cycle. By convention for nocturnally active animals, 0° represents dawn (following the subjective night, coinciding with the end of the light pulse in the short T cycle) and 180° represents dusk (preceding the subjective night, coinciding with the end of the light pulse in the long T cycle; Fig. 1). Adult male hamsters entrained to these cycles were killed in groups at 45° intervals across the circadian cycle (n = 3 animals at each of eight time points for each T-cycle group, with time points from 0° to 135° designated as subjective day and those from 180° to 315° as subjective night), and their brains were cut and SCN tissue sections processed for in situ hybridization using 35S-labeled antisense cRNA probes and film autoradiography.

To first verify that the opposite phases of locomotor rhythmicity in the short and long T cycles actually reflect opposite phases of the circadian system as a whole, and to validate our subjective day and subjective night phase assignments, we checked two additional rhythms in the killed animals. We assayed levels of the nocturnally produced hormone melatonin in pineal glands (Fig. 2A) and mRNA levels of SCN Bmal1 (known to be photo-in insensitive and rhythmically higher during the night than the day) by in situ hybridization of SCN tissue sections (Fig. 2B). These temporal profiles confirmed that the light pulse in the 23.33-h T cycle acts as dawn, marking the end of the subjective night and beginning of subjective day; and that the light pulse in the 24.67-h T cycle acts as dusk, marking the end of the subjective day and beginning of subjective night. Thus, it is the locomotor activity bout that is phased differently in the two T cycles, with offset at dawn in the 23.33-h T cycle and onset at dusk in the 24.67-h T cycle. Comparison of melatonin levels from glands pooled from subjective day with those from subjective night confirmed a significant rhythm in both T-cycle groups, with higher levels during the night (P < 0.002 and < 0.03 for 23.33-h and 24.67-h groups, respectively; Student t test; Fig. 2A). Similarly, there was a significant subjective day/night rhythm in SCN Bmal1 levels, with higher levels during the night (P < 0.02 and < 0.0001 for 23.33-h and 24.67-h groups, respectively; Student t test; Fig. 2B).

Last, to demonstrate that animals were actually entrained to the short and long T cycles, a separate set of entrained animals was released into constant darkness (DD); upon release into DD, the phase of the free-running locomotor rhythm was predicted by the phase under entrained conditions for both the 23.33-h and 24.67-h T cycles (Fig. 2C), confirming true entrainment. Of note, the free-running period in DD (τ) showed prominent aftereffects of the previous entraining cycle, i.e., it was relatively short in the 23.33-h T-cycle group (23.58 ± 0.03 h, n = 3) and relatively long in the 24.67-h T-cycle group (24.58 ± 0.04 h, n = 4) compared with the species-typical τ close to 24 h (as documented in the literature over the past few decades) (11, 12). Interestingly, τ was statistically indistinguishable from the period of the previous T cycle for the 24.67-h animals (95% confidence interval for τ of 24.45–24.71 h) but not for the 23.33-h animals (23.45–23.70 h).
SCN Per1 and Per2 mRNA Rhythms During Photoentrainment to T Cycles. Fig. 3 shows representative SCN coronal tissue sections from animals killed at 45° intervals and processed for Per1 and Per2 by in situ hybridization. As expected, SCN Per1 and Per2 mRNA levels were rhythmic in both short and long T-cycle groups, with relatively higher levels during the subjective day than subjective night, but with notable differences in localization and timing. During the 23.33-h T cycle, Per1 expression was high at 0°, with elevated signal in discrete dorsomedial and ventrolateral SCN subregions; by 45° the signal encompassed the entire dorsoventral extent of the SCN; and by 135° it was clearly present but diminished. Expression of Per2 lagged Per1, first clearly appearing at 45°, but last visible (like Per1) at 135°. During the 24.67-h T cycle, Per1 was also relatively high at 0°, but elevated signal was not seen in the ventrolateral subregion; over the course of the day the signal encompassed the full extent of the SCN and was last visible at 135°. Again, increased expression of Per2 lagged Per1, first clearly appearing at 45°, but its expression was extended (unlike Per1) to the end of the dusk light pulse at 180° and remained present but diminished at 225°. A semi-quantitative analysis of these temporal patterns for the whole SCN is shown in Fig. 4. There was no difference in either the duration or the amplitude of rhythmic Per1 expression during entrainment to the 23.33-h or 24.67-h T cycle; two-way ANOVA yielded a significant effect of time (P < 0.0001) but no effect of T-cycle group (P = 0.87) or of the interaction (P = 0.56). In contrast, both the duration and the amplitude of rhythmic Per2 expression were greater under the 24.67-h than under the 23.33-h T cycle; two-way ANOVA yielded a significant effect of time (P < 0.0001), of T-cycle group (P = 0.0001), and of the interaction (P = 0.0028).

We determined the photic dependence of Per1 expression at the end of the light pulse at 0° during 23.33-h T-cycle entrainment (dawn) and of Per2 expression at the end of the light pulse at 180° during 24.67-h T-cycle entrainment (dusk). A separate set of entrained animals was killed at 0° or 180°, either at the end of the entraining light pulses or at the same circadian phases but with the lights not turned on (n = 3 for each group). As illustrated in Fig. 5, dawn expression of Per1 at 0° during the 23.33-h T cycle did depend on the presence of light, especially and predictably in the ventral subregion, but, surprisingly, dusk expression of Per2 at 180° during the 24.67-h T cycle did not. Relative OD measurements of dawn Per1 in the ventral and dorsal SCN with lights on were significantly greater than with lights off (P = 0.002 and 0.009, respectively, two-tailed Student t test), whereas measurements of dusk Per2 in the two SCN subdivisions were no different in the presence or absence of light (P = 0.12 and 0.28, respectively, two-tailed Student t test).

Acute Nighttime Photic Induction of SCN Per1 and Per2 mRNA During T-Cycle Entrainment. Our data indicate that entrainment to short or long T cycles differentially affects rhythmic SCN Per1 and Per2 gene expression. The dawn (0°) light pulse of the 23.33-h T cycle acutely induced Per1 mRNA. Though the dusk (180°) light pulse of the 24.67-h T cycle did not acutely induce Per2 mRNA, entrainment of the clock to the long T cycle somehow altered Per2 steady-state regulation, resulting in a Per2 rhythm with increased mRNA levels during subjective day and with an extended duration of elevation. To probe the state of the circadian system under entrainment to each of the T cycles, we analyzed the dynamics of Per gene expression after a light pulse administered in the middle of the subjective night, when gene expression was low (Fig. 4); subjective-night light pulses are known to acutely induce Per1 and Per2 gene expression with different temporal profiles (13–16). An additional set of animals was entrained to short or long T cycles and then exposed to a single 30-min light pulse at 270°; animals were killed in groups (n = 3 animals at each of six time points for each T-cycle group), and their brains were cut and SCN tissue sections processed for in situ hybridization using film autoradiography and 35S-labeled antisense cRNA probes against Per1 and Per2 intronic and exonic sequences. Because splicing of introns from heteronuclear mRNA is relatively rapid, a rise in hybridization signal with the intronic probe likely reflects de novo synthesis of immature transcript; however, elevated...
Exonic expression without concomitantly elevated intronic expression suggests increased stability of mature transcript. The subjective-night light pulse induced both Per1 and Per2 gene expression in the SCN as measured by both probes, and a semi-quantitative analysis of the temporal pattern of increased expression is plotted in Fig. 6, normalized for each probe as the relative change from baseline at time 0 in darkness. For the Per1 exonic probe, two-way ANOVA yielded no effect of T-cycle group (P = 0.13), but there was an effect of time (P < 0.0001) and of the interaction (P < 0.03); the peak increase in hybridization signal occurred earlier at 60 min under the 24.67-h T cycle and later at 120 min under the 23.33-h T cycle (Fig. 6A). For the Per1 intronic probe, there was a significant effect of T-cycle group (P = 0.001), of time (P < 0.0001), and of the interaction (P < 0.03), with elevated expression at later time points under the 23.33-h T cycle (Fig. 6C). For the Per2 exonic probe, there was a significant effect of T-cycle group (P = 0.01) and of time (P < 0.0001), but no effect of the interaction (P = 0.24); hybridization signal continued to increase after the light pulse even to include the last time point (Fig. 6B). However, for the Per2 intronic probe, there was a significant effect of time only (P < 0.03), with no effect of T-cycle group (P = 0.47) or of the interaction (P = 0.20; Fig. 6D). Thus, for Per2, persistently elevated exonic expression in the 24.67-h T-cycle group was unaccompanied by elevated intronic expression, suggesting a more stable Per2 transcript under the 24.67-h T cycle.

Discussion

Entraining hamsters to a single 1-h light pulse repeated as a short or long T cycle has enabled us to analyze steady-state SCN Per1 and Per2 rhythms during differential entrainment to either simulated dawn or dusk; true entrainment under these conditions was confirmed by measuring the phases of rhythms of pineal melatonin content, SCN Bmal1 gene expression, and locomotor activity (and for the latter, its onset after animals were released into DD). This T-cycle paradigm, taken from the work of Elliott (7, 8), is distinct from previous studies of SCN Per1 and Per2 gene expression, which have relied on patterns of synchronization to complete photoperiods, responses to an acute light pulse during DD, or examination of animals genetically deficient in one or more clock genes. Our study is unique in that it analyzes the expression of such genes by cyclic light pulses rather than by complete light/dark cycles (17) that induce stable entrainment; light exposure at dawn and dusk likely represents a more accurate reflection of how entrainment takes place in nocturnal animals under natural conditions (18, 19).

As expected for both entrainment groups, SCN Per1 and Per2 mRNA levels were rhythmic, with relatively higher levels during
the subjective day than night. The expression of Per2 lagged that of Per1, so that Per1 (but not Per2) was expressed immediately at the end of the dawn (0°) light pulse of the 23.33-h T cycle, whereas Per2 (but not Per1) was expressed at the end of the dusk (180°) light pulse of the 24.67-h T cycle. Importantly, our results revealed fundamental differences in the effects of light on Per expression when entrainment was accomplished by advances or delays. The dawn (advancing) light pulse of the short T cycle acted to acutely elevate Per1 mRNA from its low baseline level characteristic of the preceding subjective night; this was especially evident in the ventrolateral subregion of the SCN, as would be predicted in hamsters (20–22). In contrast, and surprisingly, the dusk (delaying) light pulse of the long T cycle had no acute effect on either Per1 or Per2 mRNA, but entrainment of the clock to the long T cycle somehow altered Per2 steady-state regulation, resulting in a Per2 mRNA rhythm with subjective day levels of increased amplitude and extended duration. In this way, morning light advanced the onset of the Per1 mRNA rhythm, whereas evening light delayed the offset of the Per2 mRNA rhythm. We do not believe that these contrasting effects represented an artifact of the specific T cycles we selected, e.g., that one of the cycles might have been further from the species-natural τ than the other. Both cycles led to entrainment of 100% of our hamsters, and lie just within the limits of stable entrainment of hamsters to a single 1-h light pulse (with estimated 50% of animals entrained under 24.7- to 24.8-h and 23.1- to 23.2-h cycles) (8, 23).

To begin to understand the molecular mechanisms that might underlie the change in the Per2 mRNA expression pattern, we probed the state of the circadian clock in the SCN by analyzing the photoresponsiveness of Per1 and Per2 to a brief light pulse administered in the middle of the subjective night, when levels of both transcripts are at their minimum. For this experiment, we assumed that the durations of subjective day and night were compressed and decompressed equally under short and long T; we also ignored the possible complication of the animals’ locomotor activity per se, which in the long T cycle began and ended earlier than in the short T cycle (Fig. 4). Our results using intronic and exonic probes hint that Per1 transcription was sustained longer after administration of the photic stimulus during the short T cycle, although this was not associated with a T-cycle gene expression pattern. Further studies will be required to determine the expression patterns of SCN PER2 protein under T-cycle entrainment, their possible relationships to entrainment aftereffects on τ, and the potential role of behavioral activity per se on gene regulation.

Experimental Procedures

Animals. Male Syrian hamsters (Mesocricetus auratus; Charles River), 21 d old at the time of delivery to the laboratory, were housed individually in polycarbonate cages contained within well-ventilated, light-proof environmental cabinets isolated in an animal facility. Food and water were freely available and replenished at irregular hours. Light (300–400 lx at the midcage level) was provided by 15-watt cool-white fluorescent tubes mounted above the shelves and automatically controlled by a programmable timer. Animals were exposed to a 1-h light pulse presented every 23.33 h (23.33-h T cycle) or every 24.67 h (24.67-h T cycle) and maintained (entrained) for 12–16 wk unless otherwise indicated. Dim red light was used for animal care and husbandry when necessary. All behavioral experiments were conducted at the University of Massachusetts Medical School and performed in compliance with the Animal Care and Use Committees of the University of Massachusetts Medical School and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

For measurement of locomotor (wheel-running) activity, a microswitch on the outside of each cage was activated by the rotation of a 125-mm (diameter) metal running wheel; the number of switch closures per 15-min interval was automatically recorded, stored on hard disk by an IBM computer-based data acquisition system (Dataquest; Mini-Mitter), and plotted for each animal as an actogram.

Melatonin Assays. Melatonin content from individual sonicated pineal glands was determined by ELISA (IBL International), according to the protocol of von Gall et al. (38). Briefly, duplicate supernatants of each homogenized pineal were incubated overnight with a rabbit polyclonal antibody and a known amount of biotinylated melatonin. After several rinses, the bound melatonin was exposed to a colorimetric reaction and the concentration calculated.
according to a standard curve. The assay sensitivity was 1.6 pg/mL. All samples were assayed in a single assay with an intra-assay variability (SD relative to the mean) of 9.2%.

In Situ Hybridization. Animals were decapitated under dim red light. Brains were rapidly removed and frozen in 2-methylbutane cooled to −30 °C. The 15-μm-thick coronal sections through the SCN were cut on a cryostat and mounted onto slides coated with Vectabond (Vector Laboratories).

Plasmids used to generate antisense cRNA probes for hamster Per1 and Per2 (exonic probes) were generously provided by H. Okamura (Kyoto University, Japan) and S. Shibata (Waseda University, Tokyo, Japan), respectively. A PCR amplicon with RNA polymerase promoter regions was used for Bmal1 (template kindly provided by C. Weitz, Harvard Medical School, Boston, MA).

Syrian hamster Per1 intron 17 and Per2 intron 2 probes were obtained by first amplifying hamster genomic DNA with primer pairs located in the flanking exons to obtain intron sequences. Subsequently, nested primers were used to isolate an intronic fragment. For Per1, exonic primers (corresponding to nt 2215–2334 and 2454–2477 of the hamster Per1 cDNA; GenBank accession no. AY423771.1) yielded an 841-bp product containing intron 17 (GenBank accession no. JF739178). Primers based on intronic sequence were then used to amplify a 515-bp fragment (nt 154–268; GenBank accession no. JF739176), which was subcloned into Topo Dual promoter plasmid (Invitrogen) for generation of Per1 intronic sense and antisense probes. For Per2, the exonic primers (corresponding to nt 108–130 and 231–255; GenBank accession no. AF306648.1) yielded an 844-bp fragment containing intron 2 (GenBank accession no. JF739179). Nested intronic primers were then used to amplify a 625-bp fragment (nt 167–791; GenBank accession no. JF739179), which was subcloned and used to generate the Per2 intronic sense and antisense probes. Intronic probes were validated using reference SCN sections from test animals killed either in the dark or after a light pulse.

As measured by relative optical density (RD; SCN adjacent hypothalamus), hybridization intensity with each of the antisense probes was significantly increased 12 h after light exposure compared with the dark controls (P < 0.005; Student t test), whereas RD values from sections probed with the sense probes were low (near 1) and did not differ significantly from the values for the dark controls.

In vitro transcription reactions were performed in the presence of 35S-UTP with the appropriate RNA polymerase. In situ hybridization with the radiolabeled cRNA probes was performed as previously described (22, 39, 40). Serial coronal sections were hybridized with the same probe at intensities of 75 (Fig. 4C) and 120 μm (Fig. 4D). ODS of film autoradiograms were measured in ImageJ (National Institutes of Health). Templates were made for subregions or the whole SCN by taking the best-fit outline from several representative sections picked at random from the experimental samples. Background values were determined by sampling the adjacent hypothalamus of each section. RD was calculated as the OD value for the region of interest divided by the background OD value for the same section; this normalized OD was measured bilaterally for each section for 2–4 sections from each animal. The duration of elevated Per expression was calculated using onset and offset at a level midway between peak and trough values, the latter defined as the average level at the three highest and the three lowest contiguous time points, respectively.

ACKNOWLEDGMENTS. We thank Drs. Jeffrey Elliott and Roelof Hut for discussions, Dr. Charlotte von Gall for advice on melanin assays; Ms. Dorothy Lam for help with in situ hybridization; and Drs. Hitoshi Okamura, Shigenobu Shibata, and Charles Weitz for gifts of recombinant plasmids. Support for this work was provided in part by National Institutes of Health Grants R01 GM094109 (to W.J.S.), R01 NS056125 (to D.R.W), and R01 MH70516 (to H.O.D.).