Light induces c-fos and per1 expression in the suprachiasmatic nucleus of arrhythmic hamsters

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A fundamental feature of circadian rhythms is that they readily synchronize (i.e., entrain) to daily cycles of illumination and resynchronize to the photocycle when it is shifted by several hours (1, 13). One exception to this behavior has been reported for the Siberian hamster (Phodopus sungorus sungorus). When these animals are exposed to a 5-h phase delay of the photocycle, the majority of animals do not reentrain, but instead free-run with periods close to 25 h (25). We have also found that ~15% of hamsters exposed to the phase delay consistently become arrhythmic (24, 25). A much greater proportion (50–75%) of hamsters can be made arrhythmic if they are treated with an appropriately timed light pulse on the night before a phase shift or if appropriately timed light pulses are administered on two consecutive nights (22, 28). Hamsters that have arrhythmic locomotor activity patterns also lack circadian rhythms in body temperature, sleep and wakefulness, and melatonin (13, 25, 28). Arrhythmicity is induced within a few days of these light treatments and lasts indefinitely. Two laboratories have now monitored a substantial number of these animals for several months without spontaneous reemergence of circadian organization (25, 28).

A more common way to induce arrhythmicity is by long-term exposure to continuous bright light (LL). Several weeks of LL is usually required before animals become arrhythmic, and reinstatement of the LD cycle restores rhythms within a few days (4–6, 36). The most striking feature of arrhythmicity induced by a phase shift of the LD cycle compared with arrhythmicity induced by LL is that it persists in the presence of the LD cycle (25, 28). Thus the light treatments discussed above not only make Siberian hamsters arrhythmic, they also desensitize their circadian system to the restorative effects of the LD cycle. The rapid restoration of circadian rhythms by exposure to the LD cycle in animals held in LL also shows that arrhythmicity induced by LL requires continuous light exposure and is temporary, whereas arrhythmicity induced by light pulses or phase shifts of the LD cycle appears to be permanent.

These observations suggest that light pulses or phase shifts of the LD cycle that induced arrhythmicity also rendered the circadian system of these hamsters insensitive to light. A lack of photosensitivity could be manifested in the afferent inputs to the circadian pacemaker or in the pacemaker itself. The suprachiasmatic nucleus (SCN) is the primary circadian pacemaker in mammals and drives rhythms in physiology and behavior. In typical circadian rhythm studies in which animals are free-running in constant darkness (DD), light sensitivity of the SCN can be measured indirectly through light-induced phase shifts of behavioral rhythms. This cannot be done in arrhythmic animals because there is no circadian phase against which to measure a phase shift. Although SCN photosensitivity cannot be measured behaviorally in an arrhythmic animal, it can be assayed at the cellular level by examination of photic gene induction. Both per1 and c-fos genes are rapidly induced in the SCN of entrained animals by light pulses administered during the subjective night but not during the subjective day (15, 30, 31, 37). These genes are not, however, induced by light at other circadian phases. Whereas per1 is considered part of the core clock mechanism, the role of c-fos in circadian rhythms is unclear, but its rapid induction by light has been used extensively as a cellular marker for times when light can phase shift the clock (11, 26). We hypothesized that light pulses would not induce per1 or c-fos in arrhythmic hamsters because arrhyth-
mic hamsters were behaviorally unresponsive to the LD cycle and because in a previous study, we found that light pulses failed to induce expression of these genes in free-running hamsters that failed to reentrain to the LD cycle (3).

**MATERIALS AND METHODS**

**Housing conditions.** Siberian hamsters (P. sungorus sungorus) were bred from animals provided by Dr. Irving Zucker of the University of California, Berkeley, CA. Hamsters were maintained from birth in a 16:8-h light-dark (LD) cycle [lights on at 0200 Pacific Standard Time (PST)] at an ambient temperature of 22°C. Hamsters were housed two to four per cage in the animal colony and individually housed during the experiment in white polypropylene cages (30 × 17 × 17 cm; Nalgene). During experiments, hamsters were housed in six recording chambers (10 animals per chamber), each of which has its own light source and photosensor that permits illumination cycles to be recorded by computer. Animals were provided cotton batting for nesting material; food (Purina chow # 5015) and tap water were available ad libitum. All procedures involving animal care and use were approved by Stanford University’s Administrative Panel on Laboratory Animal Care.

Light fixtures illuminating both the room where light pulses were administered and the activity recording chambers contained cool white fluorescent tubes (4,100 K, Philips 40 W). Light intensity for both room light and light pulses was 10–60 μW/cm² on cage floors with water bottles, food, and cage lids in place, depending on cage location in the room or chamber and the position of the light meter photocell (International Light Model IL-140S Radiometer System) within the cage. The light sensor was pointed upward from the cage bottom for these measurements.

**Activity recording.** Activity was measured by passive infrared motion detectors mounted directly above the tip of the water bottle sipper tube. In this configuration, activity levels primarily reflect drinking behavior and locomotor activity that occurs directly under the sipper tube. These detectors have a temporal resolution of 1–2 s for successive counts of activity. Activity bouts were summed in 10-min intervals and stored on computer.

**Activity data analysis.** The presence or absence of circadian periodicity in locomotor activity was determined by a standard deviation-based periodogram analysis on the last 10 days of data for each animal immediately before brain removal (5a). Peaks in the periodogram were deemed statistically significant if they exceeded the 99.9% confidence interval limit. Activity rhythms were classified as entrained or arrhythmic after periodogram analysis. Rhythms were considered entrained if the period estimate was between 23.83 and 24.17 h and if daily rhythm onsets maintained a stable phase relationship to the LD cycle (i.e., when daily activity onsets occurred within 60 min over 10 successive days). Animals were considered arrhythmic in the LD cycle if there were no significant peaks in the periodogram in the circadian range, activity was distributed throughout the LD cycle, and daily rhythm onsets and offsets could not be identified.

**Experimental protocol.** Equal numbers of males and females were used in all groups. Experimental animals were separated and housed singly in the same photoperiod as the colony (LD 16:8, lights on at 0200 PST). Fourteen days later, the LD cycle was phase delayed by 5 h via an extension of the light phase (lights on at 0700 PST), and activity patterns were monitored for each animal. Two to four weeks after the phase shift, animals were classified as entrained or arrhythmic, as described above. The LD cycle was then terminated, and all animals were held in constant darkness (DD) for 24 h before light pulse treatment and brain removal. During DD, animals were administered a light or “dark” pulse for 30 min beginning 2 h after the projected time of dark onset. For both light and dark pulses, animals remained in their home cage but were moved to an anteroom for 30 min. Dark-pulsed animals were treated exactly the same as light-pulsed animals, except the lights were not turned on. For all hamsters, brains were extracted immediately after the 30-min pulse and prepared as described above. A separate group of animals were treated exactly as described above but were not killed after receiving a light or dark pulse. Instead, these animals remained in DD for 10 days after the pulse to determine whether our lighting protocol could restore rhythms to arrhythmic hamsters (24).

**Tissue preparation procedure.** Hamsters were decapitated at the end of a 30-min light or dark pulse [dark pulse hamsters were decapitated and brains removed under dim red light (0.5–0.8 μW/cm²)]. Brains were frozen on dry ice, then coated in embedding medium (Optimal Cutting Temperature Compound, Tissue Tek 4583) and stored at −70°C until they were sectioned coronally at 15 μm through the hypothalamic SCN region on a cryostat. Sections were thaw mounted on microslides (precleaned Superfrost Plus, VWR Scientific), fixed in 4% paraformaldehyde, dehydrated through an ethanol series, and stored at −70°C.

**35S-radiolabeled probe preparation.** Sense and anti-sense per1 and c-fos probes were made from Siberian hamster cDNA (3). RNA isolation and Northern blot analysis was done according to O’Hara et al. (17). In situ studies, Siberian hamster cDNA clones for per1 and c-fos were digested in pBlue-script (KS−) (Stratagene, La Jolla, CA) to generate a linearized DNA template. 35S-labeled anti-sense and sense probes for c-fos and per1 were transcribed from template DNA with T7 RNA polymerase and S4 RNA polymerase in a reaction containing 5 mM each of CTP, ATP, and GTP; 10 mM dithiothreitol (DTT); 1× transcription buffer (Boehringer Mannheim, Indianapolis, IN); RNasin (Promega, Madison, WI); 35S-UTP (>1,000 μCi/mmol; NEN, Boston, MA). The probe was generated from 1-μl template DNA with T7/S4 RNA polymerase, according to the manufacturer’s DIG RNA labeling kit protocol (Boehringer Mannheim). Probes were incubated with hydrolysis solution (50 mM DTT, 40 mM NaHCO3, 60 mM Na2CO3) for 1 h at 37°C, followed by treatment with neutralization solution (50 mM DTT, 5% acetic acid, 100 mM sodium acetate). Probes were then precipitated and stored at −70°C.

**In situ hybridization.** Sections were thawed to room temperature, rehydrated, incubated with 20 μg/ml proteinase K, and treated with acetic anhydride in 0.1 M triethanolamine. Dehydrated sections were hybridized for 12 h at 60°C with 5 × 10⁶ cpm/ml 35S-labeled per1 or c-fos RNA anti-sense probe or sense probe in hybridization buffer [50% formamide, 300 mM NaCl, 20 mM Tris HCL (pH 7.4), 5 mM EDTA, 10 mM NaPO4 (pH 8.0), 100 mM DTT, 10% dextran sulfate 1× Denhardt, 50 μg/ml total yeast RNA] under siliconized coverslips. Coverslips were floated off in 4× SSC at room temperature, followed by four washes in 2× SSC, DTT. Slides were rinsed in washing buffer [400 mM NaCl, 10 mM Tris HCL (pH 7.5), 5 mM EDTA] and incubated with 20 μg/ml RNAse A in washing buffer at 37°C, followed by washes in 2× SSC and 0.1× SSC. Slides were dehydrated and placed on autoradiography film (Kodak Hyperfilm MP) for direct imaging to reveal areas of RNA expression resulting from annealed β-emitting 35S-labeled probes. After 21-day autoradiography exposure, film was developed.

**In situ hybridization data analysis.** Analysis of sections was carried out using optical density (OD) analysis software (SimplePCI). SCN regions were identified on the autoradiographic image, and OD readings were taken by subtracting background OD in a region dorsal to the SCN from OD in the most densely labeled region of the SCN. We used only the most densely labeled SCN region for quantification because it provides the strongest test of our hypothesis that light would not induce gene expression in arrhythmic animals. This method provided a measure of relative optical density (ROD) for c-fos and per1 for each animal in each condition. ROD values were averaged among animals in each treatment group. Two-way ANOVA was used to independently compare ROD values of c-fos or per1 among entrained and arrhythmic animals given light or dark pulses. Tukey’s correction was applied for all post hoc pairwise comparisons. Analyses were considered significant if P < 0.05. All values are means ± SE.
RESULTS

Activity rhythms. All animals were stably entrained before the phase shift. Although most animals free-ran after the phase shift, only hamsters that were either clearly entrained or arrhythmic were administered light pulses and used for in situ hybridization (Fig. 1). Animals that were reentrained adjusted to the new phase of the LD cycle within 6–8 days and maintained nocturnal activity patterns after the phase shift. By contrast, activity was distributed throughout the day and night in arrhythmic hamsters, and periodogram analyses confirmed that circadian rhythms were absent in arrhythmic animals (P < 0.001; Fig. 1). No obvious differences in activity patterns were observed between arrhythmic animals before administration of light or dark pulses. Similarly, neither alpha nor phase angle of entrainment differed between reentrained animals that were administered light or dark pulses (P > 0.05). Some of the arrhythmic hamsters had delayed activity onsets before the phase shift, whereas reentrained hamsters did not. This was not, however, a consistent feature of arrhythmic hamsters in this study or in prior studies (23). None of the animals that remained in DD for 10 days after receiving light (n = 6) or dark (n = 5) pulses regained rhythms (Fig. 2). Periodogram analyses failed to detect any significant rhythms in the circadian range (P > 0.001) during that time.

In situ hybridization. In situ hybridization with Siberian hamster sense and anti-sense mRNA per1 and c-fos probes confirmed absence of signal for all sense probes (Fig. 3). Light pulses induced c-fos and per1 mRNA in the SCN of all entrained animals (n = 4; Fig. 3). Robust induction of c-fos and per1 was also seen in the SCN of arrhythmic animals after administration of a light pulse (n = 4; Fig. 3). Entrained (n = 4) and arrhythmic (n = 4) hamsters given dark pulses showed virtually no expression of c-fos or per1 in the SCN (Fig. 3).

Quantification of tissue sections from entrained and arrhythmic animals was done by two-way ANOVA for each gene. Only the results of the post hoc tests are reported here. The two factors in each ANOVA were rhythm (i.e., reentrained vs. arrhythmic) and pulse (i.e., light vs. dark). For both c-fos and per1, pulse was significant for arrhythmic and reentrained animals (P < 0.001), but rhythm was not significant for either gene (P > 0.05). Tissue sections from entrained and arrhythmic animals revealed significantly higher light absorbance among hamsters administered a light pulse compared with those administered a dark pulse (Tukey’s corrected t-test, P < 0.001; Fig. 4). There were no significant differences in c-fos or per1 induction among arrhythmic and entrained hamsters administered a light pulse (Tukey’s corrected t-test, P > 0.05 for each gene; Fig. 4). In none of the animals was an intermediate level of gene induction observed.

DISCUSSION

Contrary to our expectations, light induction of both c-fos and per1 in the SCN was as robust in arrhythmic hamsters as it was in entrained ones. Given that light information clearly reached the SCN, it is all the more difficult to understand why the presence of the LD cycle or exposure to DD did not restore behavioral rhythms as it does to animals made arrhythmic by exposure to LL. In other rodent species made arrhythmic by exposure to LL, the transition to DD or to an LD cycle restores rhythms within a few days. In those studies, however, animals
are typically returned to an LD cycle with 10–12 h of darkness (4–6, 36). One possibility is that, in the present study, the dark phase was too short (8 h). This limited daily exposure to darkness may be insufficient to counteract the effects of light, and animals may have responded to the 16:8 LD cycle as if they were essentially experiencing LL. This also appears to be the case for Siberian hamsters that free-run, rather than become arrhythmic, after a 5-h phase delay of the LD cycle (25). These animals tend to free-run with a mean rhythm period that is no different from hamsters housed in LL (7, 8, 19). Although these phenomena suggest that the behavior of unentrained free-running or arrhythmic hamsters bears some similarity to animals held in LL, their failure to regain rhythms in DD does not. Rather, it suggests that there are different mechanisms that underlie arrhythmicity induced by LL compared with arrhythmicity induced by a phase delay of the LD cycle. The failure of the light pulse to reinstate rhythmicity after inducing $c$-fos and $per1$ expression in the SCN is also noteworthy because it suggests that locomotor activity patterns can be dissociated from changes in clock gene expression.

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**Fig. 2.** Representative actograms of arrhythmic hamsters housed in constant darkness (DD) for 10 days. Animals received either a dark (left) or light (right; solid circle) pulse on the first full day in DD. Gray shaded areas indicate dark phase of the LD cycle and subsequent period of constant darkness. Periodogram analyses failed to detect significant rhythms in the circadian range during the 10 days before or after DD onset ($P < 0.001$).

**Fig. 3.** Representative sections of $c$-fos and $per1$ expression in the SCN of entrained and arrhythmic animals probed with Siberian hamster cDNA anti-sense and sense probes after administration of a light pulse (LP) or a dark pulse (DP). Coronal sections (top) with magnified ($\times5$) SCN region (bottom) shown for animals in each group and condition. Absence of expression was observed in sections from entrained animals probed with $c$-fos and $per1$ sense probes (right).
Arrhythmic locomotor activity patterns in the present study were probably accompanied by arrhythmicity at the level of the SCN. Previous studies have shown that loss of arrhythmicity in activity is accompanied by arrhythmicity in body temperature, sleep/wake cycles, and melatonin (13, 25, 28). Studies in which the SCN is monitored in vitro have shown in rats, golden hamsters, Siberian hamsters, and mice that when locomotor activity rhythms are lost, electrical rhythms among large populations of SCN neurons are also eliminated (13a, 14, 18, 27a). There are two primary possibilities to explain behavioral arrhythmicity at the level of the SCN when one considers that individual SCN neurons have self-sustained oscillations in electrical activity (9, 35). Individual SCN cells may have become desynchronized from one another, as is the case for arrhythmicity induced by LL (18), or individual SCN cells may have become arrhythmic. In either case, the lack of coherent output from the SCN would result in a loss of behavioral rhythms. The latter possibility may better explain our data. Light pulses induce expression of c-fos and per1 only during subjective night (11, 15, 21). Because individual SCN neurons retain circadian rhythmicity, light should induce gene expression in a single SCN neuron during its “subjective night”. In other words, a single SCN neuron should possess the same circadian rhythm in light sensitivity observed in the total population of SCN neurons. If individual SCN cells continued to oscillate completely out of phase with one another, a light pulse given at any time of day should induce gene expression in only the subset of cells that are photosensitive at that particular time. The level of gene induction should then be much less than in entrained animals in which SCN neurons are synchronized to one another. Induction of c-fos and per1 in arrhythmic animals did not, however, differ from induction in entrained ones. Our results are better explained by assuming that circadian regulation of transcription of these genes is lost in individual SCN neurons. Once the circadian gating of gene induction is eliminated, light may induce expression of c-fos and per1 in all cells regardless of time of day.

The ability to render animals arrhythmic by simple photic manipulations offers a novel method for eliminating circadian effects from studies of other physiological systems. The advantages of an arrhythmic animal model that does not require SCN lesions or other invasive procedures to eliminate rhythms are obvious. For example, studies of sleep homeostasis have used animals with SCN lesions to isolate the homeostatic response to sleep deprivation and to investigate interactions among homeostatic and circadian systems. We recently found that Siberian hamsters made arrhythmic by a 5-h phase delay of the photocycle sleep more each day and have more consolidated sleep bouts than entrained animals, but respond similarly to sleep deprivation (13). This differs from studies in other species in which animals were made arrhythmic by SCN lesions, but it remains to be determined whether SCN lesions affect sleep in Siberian hamsters as they do in other animals. Nevertheless, the use of arrhythmic hamsters with an intact SCN provides an important tool in studying sleep homeostasis.

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Siberian hamsters possess a labile circadian pacemaker that can easily be made arrhythmic by different combinations of light pulses and LD cycle phase shifts. The persistence of arrhythmia in these animals has consistently been accompanied by a complete lack of masking of locomotor activity in this and other studies (e.g., 25, 28). This lack of masking is unusual because light readily masks locomotor activity in golden hamsters and mice, even when they are made arrhythmic by surgical ablation of the SCN or by targeted disruptions in clock gene function (20, 21, 33, 34). Thus our results may have interesting implications for understanding the relationship between clock genes and behavior. Mice with targeted disruptions of both cry genes (cry1<sup>−/−</sup>, cry2<sup>−/−</sup>) express diurnal rhythms in both locomotor activity and in per2 expression in the SCN when kept in a LD cycle, but both of these rhythms were eliminated on the first day in DD (33, 34). On the basis of the correspondence between these molecular and behavioral cycles, Vitaterna et al. (34) proposed that a light-driven rhythm in SCN per2 could drive a behavioral rhythm, but our data are not consistent with this hypothesis. Our data suggest that the alternative hypothesis is more likely: locomotor activity and per2 expression are both independently driven by the LD cycle. Given that a light pulse can induce gene expression in the SCN of arrhythmic hamsters, it is also likely that the LD cycle would drive rhythms in gene expression in the SCN of these animals. The lack of masking of activity combined with the likelihood of light-driven rhythms in SCN gene expression suggests that light-driven oscillations in clock gene expression do not drive behavioral rhythms in arrhythmic mice.

Fig. 4. Intensity of c-fos and per1 mRNA hybridization in the SCN of entrained (E) and arrhythmic (AR) animals given a LP or DP. Light absorbance values, which have a direct correlation to intensity of mRNA hybridization, are reported for animals (n = 4) in each experimental condition. Note the robust induction of both c-fos and per1 in E-LP and AR-LP animals (*P < 0.001 compared with E-DP and AR-DP groups, respectively).
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