Interruption of the rat circadian clock by short light-dark cycles

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Usui, Setsuo, Terue Okazaki, and Yoshiko Honda. Interruption of the rat circadian clock by short light-dark cycles. Am J Physiol Regul Integr Comp Physiol 284: R1255–R1259, 2003; 10.1152/ajpregu.00717.2002.—Ninety male Sprague-Dawley rats were exposed to 1:1-h light-dark (LD1:1) cycles for 50–90 days, and then they were released into constant darkness (DD). During LD1:1 cycles, behavioral rhythms were gradually disintegrated, and circadian rhythms of locomotor activity, drinking, and urine 6-sulfatoxymelatonin excretion were eventually abolished. After release into DD, 44 (49%) rats showed arrhythmic behavior for >10 days. Seven (8%) animals that remained arrhythmic for >50 days in DD were exposed to brief light pulses or 12:12-h light-dark cycles, and then they restored their circadian rhythms. These results indicate that the circadian clock was stopped, at least functionally, by LD1:1 cycles and was restarted by subsequent light stimulation.

of animals to high-frequency light-dark cycles also produces disruption of circadian activity rhythms (19). The present experiment was designed to examine effects of LD1:1 on circadian rhythms in locomotor activity, drinking, and urine 6-sulfatoxymelatonin (aMT6s) excretion, and we found long-lasting circadian arrhythmicity in DD after exposure of animals to LD1:1 cycles.

METHODS

 Animals and housing. Ninety male Sprague-Dawley rats (Clea Japan, Tokyo) were housed individually in transparent polycarbonate cages (28 × 44 × 18 cm). Each cage was placed on an Animex (Muromachi Kikai, Tokyo) to detect locomotor activity. Drinking activity was measured by a drinkometer (O’Hara, Tokyo). The cage and the Animex were placed in a ventilated lightproof cabinet (71 × 46 × 35 cm) illuminated by a krypton bulb (KR100/110V40PS35WK, Toshiba Lightech, Tokyo) fixed on the inner wall of the cabinet, which was placed in a temperature-controlled (26 ± 2°C) experimental room. Illuminance measured at the center of the cage was ~100 lx during the light (L)-period and 0 lx during the dark (D)-period. Laboratory chow and water were given ad libitum throughout the experiment. Cumulative counts of drinking and locomotor activities were continuously recorded at 5-min intervals. Food was replenished, and the cage and wood chips were changed under dim red illumination (<0.5 lx) once per week. The experimental protocols were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute for Neuroscience.

 Procedures. Rats were adapted to the experimental condition of 12:12-h light-dark cycles (LD12:12) for 11–24 days. Then they were exposed to LD1:1 cycles for 50–90 days until their circadian rhythms were abolished. Thereafter the animals were released into constant darkness (DD). The rats that displayed arrhythmic behavioral patterns for >50 days in DD were exposed to light pulses (300 lx, 30 min) and/or LD12:12.

 Urine collection and measurement of aMT6s. Under LD12:12 and LD1:1 conditions, 11 rats were transferred to metabolic cages (20 × 20 × 20 cm) for 3 days to collect urine for aMT6s analysis and thereafter were returned to polycarbonate cages. Rats were housed individually in metabolic cages with transparent acrylic tops and lighting conditions similar to those in the polycarbonate cage. After an adaptation period of 24 h, sampling was conducted for 48 h during which food and water were provided ad libitum. Each metabolic chamber was equipped with a funnel system, which separated urine from feces. Urine was collected every 3 h, the volume was determined to the nearest 0.1 ml, and all samples were frozen at −20°C for later analysis. After exposure

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to DD, 5 of 11 rats recovered their circadian rhythms within 2 wk. The remaining six rats that displayed behavioral arrhythmicity were transferred to metabolic cages to collect urine. Two of six rats displayed circadian activity rhythms immediately after the return from metabolic cages. The remaining four rats showed behavioral arrhythmicity for at least 1 wk after the return from metabolic cages. These four rats were transferred into metabolic cages after they recovered their circadian activity rhythms in DD. Then urine samples from these four rats under four different conditions were subjected to aMT6s analysis. Urine samples were centrifuged at 2,500 g for 10 min to remove solid material. All urine samples were assayed in duplicate using 125I-labeled aMT6s tracer and antibody from Stockgrand (ALPCO) according to Aldhous and Arendt (1). Results were expressed in nanograms produced per hour. Data obtained during the rhythmic state in DD were plotted against internal time where the midpoint of the activity period of the free-running activity rhythm was set to 0 h (5).

Statistical analysis. Although drinking and locomotor activity data behaved similarly throughout the experiment, we analyzed locomotor activity data because locomotor activity recorded by Animex was generally more distinct in the onset and offset of the activity period than was drinking activity.

RESULTS

When rats were placed in LD1:1 cycles, they showed free-running circadian rhythms with periods >24 h. However, these rhythms gradually disintegrated and turned into diffuse or ultradian behavioral patterns without circadian rhythmicity. Circadian arrhythmicity elicited by LD1:1 continued in complete darkness. After exposure to DD, 44 (49%) and 22 (24%) of 90 rats remained arrhythmic for more than 10 and 20 days, respectively. In 83 rats, however, circadian behavioral rhythms reappeared spontaneously, without any stimulation.

Seven (8%) rats showed arrhythmic behavior for >50 days in DD (Figs. 1 and 2). One of the seven aperiodic rats that was exposed to LD12:12 cycles recovered its circadian rhythm. The remaining arrhythmic rats were exposed to a light pulse, and four rats recovered...
their circadian rhythms (Fig. 1). One of two rats that remained arrhythmic after light pulse stimulation re-
stored its circadian rhythms after exposure to a second light pulse, but another rat did not. The rat remained arrhythmic for 214 days in DD and was subsequently transferred to LD12:12 cycles, where it showed behav-
ioral circadian rhythms entrained to the light-dark cycle. After subsequent exposure to DD, its circadian rhythm strayed from the initial synchronization to the light-dark cycle (Fig. 2). All the animals that had shown circadian arrhythmicity for >50 days restored their circadian rhythms after exposure to either light pulses or LD12:12 cycles.

Under LD12:12 cycles, each animal had a clear circadian rhythm of urinary aMT6s secretion with peak during the dark period (Fig. 3A). Under LD1:1 cycles, rats displayed arrhythmic behavioral patterns, and levels of aMT6s remained very low throughout the 48-h period of measurement (Fig. 3B). When the animals displayed arrhythmic behavioral patterns in DD, aMT6s excretion remained consistently very low as under LD1:1 cycles (Fig. 3C). In contrast, animals that had recovered their circadian behavioral rhythms in DD showed clear circadian rhythms of aMT6s secretion with peaks in the subjective night (Fig. 3D).

**DISCUSSION**

The present results clearly demonstrate that arrhythmic behavior elicited by LD1:1 was not due to the masking effect of light, because behavioral arrhythmicity continued in DD for long periods (up to 214 days). As far as we know, such long-lasting circadian arrhythmicity in DD has not been reported in rats with the intact suprachiasmatic nucleus (SCN), the main clock for the circadian rhythm in mammals (11). During LD1:1 and DD, behavioral arrhythmia was associated with constant suppression of urine melatonin metabolite excretion. Similar suppression of pineal melatonin synthesis has been reported in rats with lesions in the SCN (13). These results suggest that regulation by the circadian clock was lost in the animals during circadian arrhythmicity in DD.
Circadian arrhythmicity has been reported in several species of animals exposed to LL for long periods (3, 8, 15). These arrhythmic animals restored their circadian rhythms after several days in DD (9), whereas rats exposed to LD1:1 remained arrhythmic for long periods in DD. The discrepancy in results from LL and LD1:1 experiments may be due to different responsiveness of the circadian system to light-dark transition. For animals kept in LL, transition from LL to DD may be a drastic stimulus for the circadian system. On the other hand, animals kept in the LD1:1 had been repeatedly exposed to light-dark transition, and their circadian pacemakers may be less sensitive to the stimulus.

The present study indicates that the circadian pacemaker was functionally stopped by LD1:1. However, the mechanisms underlying the behavioral and hormonal arrhythmicity remain unclear. There are at least three explanations for overt arrhythmicity in DD. First, it may be due to the cessation of circadian oscillation, implying that the oscillator has been brought to singularity and the amplitude of the oscillator is reduced (22). It has been reported that the electrical activity of SCN neurons of the hamster that displayed arrhythmic behavior in LL did not show circadian rhythms in vitro (12). However, the finding does not necessarily indicate the disruption of circadian oscillation, because preparation of the SCN for electrical recordings in vitro may cause drastic changes in the state of the pacemaker and because the electrical activity of the SCN can be dissociated from circadian oscillation (16). The second, more likely, explanation is that overt arrhythmicity may be caused by a desynchronization within multiple oscillators. It has been suggested that individual neurons in the SCN have circadian oscillators (10, 21), which may become uncoupled under some conditions. Third, it may be due to a loss of coupling between the oscillator and output pathways that are involved in generation of overt phenomena. The third explanation seems unlikely because three independent output rhythms (locomotor activity, water intake, and melatonin secretion) of the oscillator were equally affected. To test these possibilities, however, monitoring behavior of the circadian oscillator itself is required.

Mice mutant in the gene Clock show characteristic circadian rhythms. When Clock/Clock mice are placed in DD, they display free-running circadian rhythms with very long periods during several days, and then they become arrhythmic (20). In LL and LD1:1, rats initially show free-running activity rhythms with periods much longer than 24 h and then become arrhythmic. The similar patterns of development of arrhythmicity suggest the similarity of mechanisms underlying overt arrhythmicity. Recently, Nakamura et al. (14) revealed that SCN neurons from Clock/Clock mice showed circadian firing rhythms with long periods. The authors interpreted that the loss of behavioral rhythms in Clock/Clock mice was due to the uncoupling of population oscillators.

In the present study, duration of circadian arrhythmicity in DD varied markedly among the animals. Duration of arrhythmicity in DD may depend on uncontrolled factors. It may be an individual difference within Sprague-Dawley rats used in the experiments. Further studies are necessary to elucidate factors determining duration of arrhythmicity in DD.

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Fig. 3. Profiles of urinary 6-sulfatoxymelatonin (aMT6s) excretion of individual animals measured during LD12:12 cycles (A), LD1:1 cycles (B), DD without circadian behavioral rhythms (C), and DD with free-running behavioral rhythms (D). Four different bars represent data from 4 individual animals. Black horizontal bars indicate the dark period. For the rhythmic state in DD (D), internal time (InT = 0 h) was set to coincide with the midpoint of the activity period.
REFERENCES