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Restoration of Self-Sustained Circadian Rhythmicity by the Mutant *Clock* Allele in Mice in Constant Illumination

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Abstract Mice mutant for the *Clock* gene display abnormal circadian behavior characterized by long circadian periods and a tendency to become rapidly arrhythmic in constant darkness (DD). To investigate whether this result is contingent on the absence of light, the authors studied the circadian behavior of homozygous *Clock* mutant mice under conditions of both constant light and DD. Fourteen of 15 *Clock/Clock* mice stayed rhythmic in constant light of 70 to 170 lux, where 10 of 15 wild-type mice became arrhythmic. In contrast, only 5 of 15 *Clock/Clock* mice and 15 of 15 wild-type mice remained rhythmic after 60 cycles when released in DD (dim red light of < 1.5 lux) after 8 days of entrainment. The restoration of self-sustained rhythmicity by the *Clock* allele cannot be attributed to reduced sensitivity of the system to light. It underscores the fact that self-sustainment is not a secure guide to functional organization.

Key words circadian clock, constant illumination, *Clock*, mouse mutants, circadian rhythmicity

Self-sustained rhythmicity in constant conditions is considered a key functional property of circadian oscillators (e.g., Pittendrigh, 1981). Self-sustainment is, however, often restricted to constant darkness (DD) or to low levels of continuous illumination (LL). In higher light intensities, arrhythmicity usually ensues (Aschoff, 1960; Daan and Pittendrigh, 1976). Arrhythmicity of mammalian circadian systems in LL is neither physiologically nor theoretically well understood. The level-threshold model of Wever (1966) attributed the phenomenon to the increasing effect of LL on the level of a state variable. The recent identification of genes involved in the generation of mammalian circadian rhythms (King and Takahashi, 2000; Lowrey and Takahashi, 2000) makes it possible to clarify how particular gene products might be

involved in this response to LL conditions. The arrhythmicity response may help unravel the role of these genes in the circadian clock mechanism. So far, there are no studies on the effect of circadian gene mutations on the behavior of mice in LL.

The *Clock* gene in mice encodes a basic helix-loop-helix protein with two basic PER-ARNT-SIM domains. The latter may be an important structural feature of a subset of genes involved in photoreception and circadian rhythmicity. Mutation of the *Clock* gene has been reported to interfere with self-sustainment of the circadian activity rhythm (Vitaterna et al., 1994). Homozygous *Clock* mutant mice rapidly lose rhythmicity within 5 to 15 cycles in DD. Heterozygous mutants retain self-sustainment, although with an extended period length: on average 24.4 h compared

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to 23.9 h in wild-types. The rhythmicity of *Clock/Clock* mice in DD can temporarily be restored by a 6-h light pulse (Vitaterna et al., 1994). The loss of rhythmicity and period alteration in *Clock/Clock* mice can be restored by overexpression of the *Clock* transgene by bacterial artificial chromosome complementation as well (Antoch et al., 1997), proving that *Clock* is an integral component of the circadian system. In chimeric *Clock* mice, circadian behavior spans the range from WT-like phenotype to *Clock* mutant-like phenotype and the behavioral expression of the mutation is associated with its expansion in the suprachiasmatic nucleus (SCN) neuroanatomy (Low-Zeddes and Takahashi, 2001).

In this study, we address whether the interference of the *Clock* allele with circadian self-sustainment is contingent on the illumination level in constant conditions.

METHODS

We used *Clock* mutant mice back-crossed to the original wild-type for 9 to 12 generations since the original mutagenesis (Vitaterna et al., 1994). Fifteen male homozygous *Clock* mutant and 15 wild-type C57BL/6J mice (age ~10 months) were housed individually in 25 × 25 × 40 cm cages, with food and water provided ad libitum. Spontaneous locomotor activity was recorded with running wheels (14 cm in diameter) connected to an event recording system storing wheel revolutions in 2-min intervals. Mice were entrained for 8 days in light:dark (LD) 12:12 and then exposed to LL for 90 days. Animals were placed randomly with respect to light intensity (125 to 400 mW/m² at the cage floor level; white fluorescent tube light 36W/85) and were kept in the same position during the entire course of the experiment. After the episode in LL, the mice were reentrained for 8 days to LD 12:12 and subsequently kept for 90 days in dim red light (DD) (< 2 mW/m², λ_{max} = 773 nm). Temperature was maintained at 23 ± 1 °C.

The 90-day actograms obtained in constant conditions were split up into 15-day sections for analysis. Activity data from each section in each individual were subjected to chi-square periodogram analysis (Sokolove and Bushell, 1978) for the assessment of significant circadian periodicity and determination of period length.

To test the light sensitivity and phase response of the circadian system of the *Clock/Clock* mice, 13 *Clock/*

Clock and 13 +/+ mice in DD were exposed to a 1-h light pulse of 5600 lux at InT 20 and 10 days later at InT 4. (We use the notation InT [internal time] according to Daan et al. [2002]. InT runs from 0 to 24 h, with InT 0 being the phase of the rhythm coinciding in an LD cycle with mid-dark, i.e., external time [ExT] 0.) The mutant mice were made rhythmic by a prior 6-h light pulse (Vitaterna et al., 1994) to enable phase shift assessment.

RESULTS

Figure 1 shows typical examples of the actograms obtained under LL and DD for one wild-type (+/+) and one mutant (*Clock/Clock*) mouse. Both genotypes did entrain well to LD 12:12. The wild-type mouse in Figure 1C shows robust circadian locomotor activity in DD, whereas it loses rhythmicity gradually in LL (Fig. 1A). The actograms in Figures 1B and 1D are plotted on a 29-h time base, as the *Clock/Clock* mice displayed much slower rhythmicity in both LL and DD. In DD, the mutant mouse lost rhythmicity around day 28 (Fig. 1D), as described previously (Vitaterna et al., 1994). Surprisingly, however, its circadian system remained fully rhythmic throughout the 90 days in LL (Fig. 1C).

The numbers of mice with significant circadian rhythms according to chi-square periodogram analysis during the initial 15 days and the final 15 days in LL and DD are shown along with their corresponding period lengths in Table 1. After 75 days in LL, most (12 of 14) *Clock/Clock* mice still displayed self-sustained circadian rhythmicity, whereas only 5 of 15 +/+ mice had discernible circadian rhythms (χ^2 = 8.19, p < 0.01). After 75 days in DD, significantly fewer *Clock/Clock* mice remained rhythmic (χ^2 = 11.63, p < 0.005) compared to +/+ mice (5 of 15 *Clock/Clock* vs. 14 of 15 +/+).

The average period length of *Clock/Clock* mice in DD was 29.4 ± 0.4 h, indistinguishable from the period length in LL (29.4 ± 0.5 h). +/+ mice in DD had a period length of 24.0 ± 0.2 h, significantly different from the period length in LL (25.3 ± 0.3 h) (p < 0.001). The initial period length of +/+ mice that became arrhythmic in LL was significantly longer than the initial period length of +/+ mice that remained rhythmic throughout the entire 90 days in LL (p < 0.001).

In both genotypes, wheel-running activity was strongly reduced in LL compared to DD, but in LL *Clock/Clock* mice were significantly (p < 0.01) more active than the +/+ mice. Average activity of +/+ mice in DD

Figure 1. Actograms of a +/+ mouse in constant light (LL) (A) and in constant darkness (DD) (C, same individual) and a *Clock/Clock* mouse in LL (B) and DD (D, same individual). In each record, the initial 8 days of entrainment to light:dark 12:12 are followed by 90 days of LL or DD. Actograms were double plotted on a 24-h time basis in wild-type mice and on a 29-h time basis in *Clock/Clock* mice.

decreased to ~60% of the activity in LD compared to a decrease to ~30% in *Clock/Clock* mice ($p < 0.05$).

A light pulse at InT 4 evoked a phase advance in both genotypes. In *Clock/Clock* mice, the average

Table 1. Rhythmicity, circadian period length, and amount of daily activity of *Clock/Clock* and *+/+* mice.

	<i>+/+</i>			<i>Clock/Clock</i>			<i>p</i>
	<i>Mean</i>	<i>SD</i>	<i>n</i>	<i>Mean</i>	<i>SD</i>	<i>n</i>	
Circadian period τ (h)							
LD	23.9	0.00	15	23.9	0.08	14	<i>ns</i>
LL days 1-15	25.5	1.03	10	30.3	0.48	10	< 0.001
LL days 75-90	25.1	0.35	5	28.5	2.86	12	< 0.001
DD	23.9	0.02	15	23.9	0.13	15	<i>ns</i>
DD days 1-15	24.0	0.20	15	29.1	0.21	14	< 0.001
DD days 75-90	24.0	0.17	14	30.0	1.19	5	< 0.001
Test period length days 75-90	<i>p</i> < 0.001			<i>ns</i>			
Activity (wheel revolutions/day)							
LD	4419	2014	15	4375	2142	14	<i>ns</i>
LL days 1-15	408	305	15	538	425	15	<i>ns</i>
LL days 75-90	55	38	15	153	121	15	< 0.01
DD	4842	1735	15	2898	1210	15	< 0.01
DD days 1-15	3748	1735	15	2892	1462	15	<i>ns</i>
DD days 75-90	2723	2562	15	1245	706	15	< 0.05

NOTE: LD = light:dark, LL = constant light, DD = constant darkness. In the upper half of the table, *n* indicates the number of rhythmic animals (according to chi-square periodogram analysis) out of 15. The last column indicates *p* values for differences between means of wild-type and *Clock* mutant mice.

Figure 2. Phase shifts of *+/+* and *Clock/Clock* mice by a 1-h 5600-lux light pulse at internal time (InT) 20 and internal time 4 (error bars indicate SEM).

advance shift (8.4 ± 2.7 h) was significantly larger than in *+/+* mice (1.2 ± 1.0 h; two-sample *t* test, $p < 0.005$). A light pulse at InT 20 resulted in an average phase delay of 1.6 ± 0.8 h in *+/+* mice and a delay of 2.7 ± 1.6 h in *Clock/Clock* mice (two-sample *t* test, $p < 0.05$) (Fig. 2).

DISCUSSION

The data reported here unambiguously show that the *Clock* allele produced by mutagenesis (Vitaterna et al., 1994) does not interfere with the expression of self-sustained rhythmicity under all conditions. Although it does lead to loss of circadian rhythmicity in homozygous mutants in DD, it rescues self-sustained

rhythmicity in LL, where the circadian system of wild-type mice tends to become arrhythmic.

The first question one might ask with respect to behavioral arrhythmicity is whether it reflects an arrhythmic pacemaker, or a rhythmic pacemaker losing its control over the behavioral pattern. In short, is arrhythmicity evoked by alterations in the core oscillator or in the clock-controlled output? From recent work by Nakamura et al. (2002), we know that individual neurons in SCN organotypic slices, as well as dispersed cell cultures derived from *Clock/Clock* mice, retain their circadian rhythmicity in spike frequency, along with the altered circadian cycle length. This means that the circadian machinery itself is not stopped at the cellular level by the mutation. It does not exclude the possibility that the pacemaker as a whole has lost circadian self-sustainment by interference with the mutual coupling of the individual neurons. Thus, under the central hypothesis, pacemaker arrhythmia may be attributed to suppressed neuronal coupling (between *Clock/Clock* neurons); under the peripheral hypothesis, it may be attributed to suppressed rhythmicity of a key pacemaker output factor such as vasopressin, or altered transcription of double-binding protein (Ripperger et al., 2000; Yamaguchi et al., 2000). Although it is too early to distinguish between the possibilities of core oscillator versus output arrhythmicity, light intensity clearly must differentially affect the mechanism leading to arrhythmicity in wild-type and *Clock* mutant pacemakers.

Mutant CLOCK protein from the dominant-negative *Clock* allele can form heterodimers with BMAL1

that bind to DNA but fail to activate direct transcription of *mper1* (Gekakis et al., 1998). This might suggest that the activation of *mper1* transcription is not crucial for the maintenance of rhythmicity in LL. According to Shearman and Weaver (1999), photic induction of *Per* gene expression, although still present, is quantitatively reduced in *Clock/Clock* mice. This may reduce sensitivity of the circadian system to light, since basal levels of *mPer* gene expression in the dark are unaffected by the mutation, as other clock genes (e.g., *mCry1* and *mCry2*) do show lower basal expression levels (Kume et al., 1999). One might therefore hypothesize that the restoration of self-sustained rhythmicity in LL by the clock mutation is attributable to a loss of sensitivity to light compared to the wild-type circadian system. There are several arguments against this proposition. First, light pulses against a DD background are able to restore circadian rhythmicity temporarily in homozygous mutants (Vitaterna et al., 1994). Second, *Clock/Clock* mice that are made rhythmic in DD by such strong light stimuli, and subsequently exposed to a light pulse of 5600 lux for 1 h at InT 20 and InT 4, show large phase delays and advances, respectively—larger than wild-type mice (Fig. 2). This is consistent with similar previous findings by Low-Zeddies and Takahashi (2001). Third, the initial period length of *Clock/Clock* mice is increased in LL compared to DD. All these facts are incompatible with the idea that the *Clock* mutation has lost sensitivity to light.

Roenneberg and Foster (1997) raised the possibility that the *Clock* phenotype results from a defect in a gene that mimics the effect of LL on the clock rather than a defect in the clock itself. This now seems unlikely, since the genetic defect counteracts rather than reinforces the effect LL has on wild-type mice. Hence, we have to consider other mechanisms to explain the different responses of clock mutant and wild-type mice to LL. One possibility is that suppression of rhythmicity in wild-type mice is attributable to the strong suppression of behavioral activity. Possibly the degree of self-sustainment of the central pacemaker is partly dependent on feedback from activity. In homozygous clock mutants, overall activity is suppressed by LL to a lesser extent than in wild-type mice. In this respect, it is also of interest that behavioral activation during the subjective day induces opposite phase shifts in *Clock/+* mice compared to those in wild-type individuals (Challet et al., 2000).

Whatever is the mechanism leading to the rescue of sustained circadian rhythmicity in LL by the *Clock*

mutation, it highlights an important functional consideration. Circadian self-sustainment in itself is apparently not a reliable guide to selective advantage in natural selection. Self-sustainment may vary between conditions and between intact and genetically modified pacemakers. Its occurrence may provide us with clues about the mechanism; it will not always discriminate between fully functional and functionally inferior biological clocks.

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