Phase Responses to Light Pulses in Mice Lacking Functional *per* or *cry* Genes

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Abstract The phase-resetting properties of the circadian system in mice with a functional deletion in *mCry1*, *mCry2*, *mPer1*, or *mPer2* were studied in 2 experiments. In experiment 1, $mCry1^{-/-}$ and $mCry2^{-/-}$ mice as well as $mPer1^{Brdm1}$ and $mPer2^{Brdm1}$ mutant mice were exposed to 15-min light pulses during the 1st cycle following entrainment, either early (external time [ExT] 20) or late (ExT 4) in the subjective night. In experiment 2, a full PRC was measured for all these strains by exposure to light pulses of the same duration and intensity in free-running conditions in constant darkness. Directly after entrainment (experiment 1), $mPer1^{Brdm1}$ animals did not show significant phase advances by a light pulse in the late subjective night (ExT 4), as in the study by Albrecht et al. In the same experiment, $mPer2^{Brdm1}$ mice became arrhythmic too frequently to reliably measure their phase responses. Mice with a targeted gene disruption in *mCry1* or *mCry2* showed increased phase delays compared to wild type after exposure to a light pulse in the early subjective night (ExT 20). Otherwise, phase shifts were not significantly affected. In free run (experiment 2), all genotypes did show phase advances and phase delays. The $mPer2^{Brdm1}$ mutant PRC was above the $mPer1^{Brdm1}$ mutant and wild-type PRC (i.e., less delayed and more advanced) at most circadian phases. The $mPer1^{Brdm1}$ mutant PRC was not distinguishable from the wild-type PRC. The $mCry2^{-/-}$ mice showed much smaller phase delays than did $mCry1^{-/-}$ mice in the subjective evening (delay phase). In general, $mPer2^{Brdm1}$ mutant mice were more accelerated by light compared to $mPer1^{Brdm1}$ and wild-type control mice, whereas $mCry1^{-/-}$ mice were more delayed by light than were $mCry2^{-/-}$ mice.

Key words circadian clock, phase resetting, Cry1, Cry2, Per1, Per2, PRC

The notion that the circadian pacemaker in nocturnal rodents is built on 2 components with opposite responses to light has a long history (Pittendrigh and Daan, 1976b; Ilnerová and Vanecek, 1982; Wehr, 1997). This hypothesis gained new interest when Jagota et al. (2000) demonstrated that at the physiological level, there are indeed 2 subsystems in the SCN that respond differentially to changing day length. Together with the dual nature of the genetic makeup of the molecular mechanism, these findings led Daan et al. (2001) to postulate 2 genetic components in the core mechanism. They derived a series of predictions on the behavior in rodent systems in which one or the other component has been genetically deleted. According to

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the hypothesis, *Cry1* and *Per1* are involved in a component of the system that is accelerated by light and decelerated in darkness, in which the *Per2* and *Cry2* genes are involved in a component that is decelerated by light and accelerated by darkness. These properties were expected to be phenotypically reflected in free run in DD, in constant light, and in phase responses of the circadian system by light pulses in mice with a functional deletion in either of these genes.

The hypothesis was partly inspired by and based on a study by Albrecht et al. (2001), who demonstrated absence of phase advances in *mPer1* mutant mice and absence of phase delays and increased phase advances in *mPer2* mutant mice after exposure to light pulses early (ZT 14) and late (ZT 22) in the subjective night, respectively. These light pulses were applied just after entrainment in a so-called type II protocol (Aschoff, 1965). In such a protocol, the phase prior to the light pulse is derived from the rhythm under LD conditions and hence subject to masking influences, whereas there is no masking in the free run after the light pulse. This may affect the measurement of the phase shift. In addition, different circadian genotypes may have systematically deviating phase angle differences with the LD cycle. It is therefore desirable to evaluate the phase shifts relative to an unpulsed control experiment and to obtain a precise quantitative evaluation of the shift. In this study, this protocol has been applied (in experiment 1) to $mCry1^{-/-}$ and $mCry2^{-/-}$ mice and, simultaneously, in a repeat of Albrecht et al.'s work, to $mPer1^{Brdm1}$ and $mPer2^{Brdm1}$ mice.

Suppression by a genetic disturbance of the induction of either phase delays or phase advances by light pulses given at 2 time points in the cycle does not prove that all phase delays or advances are suppressed. Such suppression might in fact also be interpreted as resulting from a shift in the PRC relative to the zeitgeber, as expected if the circadian period is affected. Therefore, we obtained in addition a full PRC in a free-running situation under constant darkness (DD), a type I protocol (Aschoff, 1965). This was done in experiment 2 in all genotypes (except wild type with the same background as $mCry1^{-/-}$ and $mCry2^{-/-}$), with light pulses of the same duration and intensity as in experiment 1. The results prove that all 4 genotypes retain the capacity for both advancing and delaying responses to light pulses while at the same time revealing interesting differences between the genotypes in resetting behavior.

METHOD

The $mCry1^{-/-}$, $mCry2^{-/-}$, $mPer1^{Brdm1}$, and $mPer2^{Brdm1}$ mice have been described previously (van der Horst et al., 1999; Zheng et al., 1999). They were generated in a hybrid C57BL/6 × 129ola (*mCry* mice) and C57BL/6 × 129 SvEvBrd genetic background (*mPer* mice). Animals were housed individually in 25 × 25 × 40-cm cages, with food and water ad libitum in a sound-attenuated and climatized room with 90 cages. 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. Temperature was maintained at 23 ± 1 °C throughout the 2 experiments.

EXPERIMENT 1: PHASE RESPONSES IN ENTRAINMENT

In this study, 90 mice, all males (age, 1-2 months), were used: 15 homozygous *mCry1* ($mCry1^{-/-}$) mutant mice, 15 homozygous *mCry2* mutant mice ($mCry2^{-/-}$; van der Horst et al., 1999), 15 wild-type mice with the same background as the *mCry* mutant mice, 15 *mPer* mutant mice ($mPer1^{Brdm1}$), 15 *mPer2* mutant mice ($mPer2^{Brdm1}$; Zheng et al., 1999), and 15 wild-type mice with the same background as the *mPer* mutant mice. The experiment was designed to repeat the study by Albrecht et al. (2001) on phase shifts in response to light pulses immediately following entrainment by an LD 12:12 cycle in the $mPer^{Brdm1}$ mutant mice, while simultaneously expanding the analysis to the *mCry* gene–targeted mutant mice.

All mice were entrained for 14 days in LD 12:12. They were then released into constant darkness (DD) except for a light pulse presented in the 1st cycle during the subjective night at external time (ExT) 20 (which in LD 12:12 equals ZT 14; see Daan et al., 2002). This was followed by 14 days of free run in DD. The procedure was repeated once with a light pulse at ExT 4 (ZT 22) and once without a light pulse. This last treatment was done to assess the initial phase in a control situation after the transition from LD to DD. All light pulses had a duration of 15′ and intensity of 480 lux (700 mW/m^2 at the cage floor level; white fluorescent tube light 36W/85). All cages had equal distance to their light source; light pulse intensity was checked for all cages to deviate no more than ±140 lux (200 mW/m^2) at the cage floor level.

EXPERIMENT 2: PHASE RESPONSES IN FREE RUN

In this study, 75 mice, all males (age, 1-2 months), were used: 15 homozygous *mCry1*$^{-/-}$ mice, 15 homozygous *mCry2*$^{-/-}$ mice, 15 homozygous *mPer1*^{Brdm1} mice, 15 homozygous *mPer2*^{Brdm1} mice, and 15 wild-type mice with the same background as the *mPer* mutant mice. At the time we did this experiment, we had insufficient wild-type mice with the appropriate background for the *mCry* mutant mice available, and therefore *mCry*$^{-/-}$ mice results can only be compared between the *mCry1*$^{-/-}$ and *mCry2*$^{-/-}$ strains. All mice were entrained for 14 days in LD 12:12 and then left in constant darkness (DD) for 176 days, interrupted only by brief light pulses with equal duration (15′) and intensity (480 lux) as in experiment 1, followed by 12 days of free run. Because of the slight intraindividual variations in circadian period length within each genotype and subsequent differences in phase shifts by consecutive light pulses, the light pulses eventually became spread over the entire circadian cycle, allowing the construction of a full PRC for each of the 5 genotypes. All *mPer1*^{Brdm1}, *mCry1*$^{-/-}$, and *mCry2*$^{-/-}$ mice were exposed to 11 light pulses. The *mPer2*^{Brdm1} mice in our lab often become arrhythmic in constant darkness, although rhythmicity may recur spontaneously following a light pulse. Therefore, this strain initially yielded insufficient data for the reconstruction of a complete PRC. The experiment was therefore extended with another batch of 30 *mPer2*^{Brdm1} mice that were exposed to 4 additional light pulses.

ANALYSIS

For both experiments, phase shifts were calculated by applying a new quantitative computation method. This method uses custom-designed software, as specified in detail in the appendix. Briefly, what the software package does is calculate the phase of the circadian activity rhythm (in internal time [InT]) at exactly the time of the light pulse on the basis of forward extrapolation from the rhythm during cycles –10 to –1 (before the light pulse). Then, it calculates the phase at that time again on the basis of backward extrapolation from the rhythm during cycles 3 to 12 (after the light pulse). The difference between the 2 computed phases at exactly the same time at the onset of the light pulse is the actual phase shift. It is given a positive sign (advance) when the second phase calculation yields a smaller phase angle than does the first. It is given a negative sign (delay) when the second phase calculation yields a larger phase angle than does the first. Activity data from the first 48 h after each light pulse were omitted to avoid potential transient τ values evoked by the light pulse.

The 2 phase angles are calculated by first determining the period before (τ_1) and after (τ_2) the light pulse through periodogram analysis and by defining different phase markers on the wave form of the time series stacked with these periods. Activity onset was the most precise phase marker, as is usual in running-wheel data (see Daan and Oklejewicz, 2003), and was used for phase definition. Activity onset was defined as InT 18.

RESULTS

Experiment 1

The 1st experiment was designed to establish the phase shift induced by a 15′ light pulse at 2 different phases in the 1st cycle following entrainment to an LD 12:12 cycle. The protocol was an exact copy of the protocol used by Albrecht et al. (2001) and is known as a type II protocol (Aschoff, 1965; Mrosovsky, 1996). The phase at the time of the light pulse is assessed by extrapolation from the (entrained) rhythm before the light pulse and back extrapolation from the (free-running) rhythm after the pulse.

The difficulty with this protocol is that the activity onset under entrainment is affected by masking and therefore tends to occur at a later circadian phase than does the activity onset in the subsequent free-running situation (see Fig. 1). This is potentially different between genotypes, since mutations of circadian genes tend to elicit different free-running periods and hence different phase angles in entrainment (Fig. 2). To accommodate this difference, we also analyzed a release into free run without a preceding light pulse, as was also done by Albrecht et al. (2001).

The phase shifts obtained in this protocol were thus defined as the intraindividual differences in phase during free run following the light pulse and the phase without a light pulse. This procedure did lead to loss of data when animals in the latter control situation became arrhythmic—as often happened in *mPer2*^{Brdm1} (Oster et al., 2002)—and no phase could be

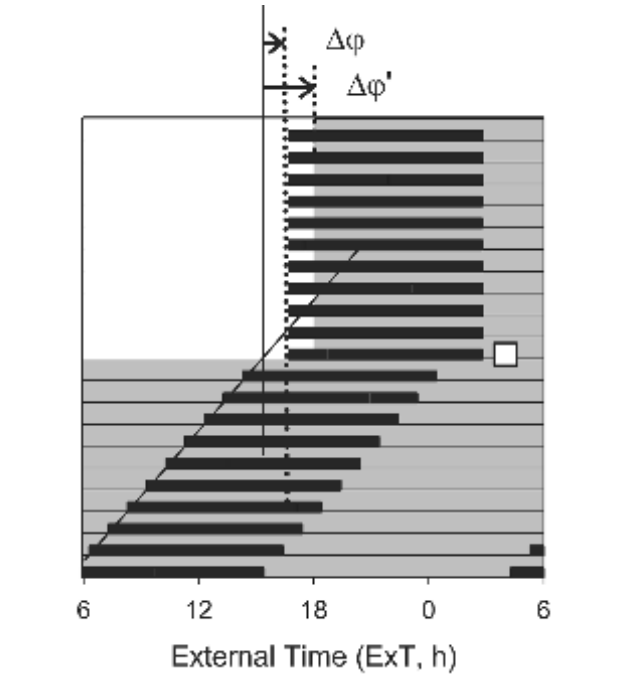

Figure 1. Application of an advancing light pulse in the subjective morning in a type II protocol (Aschoff, 1965). Δφ measured from free-running activity onset relative to prior activity onset (Δφ) and relative to prior lights-off (Δφ') yields different values when mice start activity before lights-off.

established. Examples of behavioral shifts are presented in actograms in Figure 3; the resulting average phase shifts for the *mCry* genotypes are plotted in Figure 4.

In response to the light pulse at ExT 20, *mPer1*^{Brdm1} mice did show a small average delay phase shift of –1.2 h (SEM 1.1 h). The advance phase shift following a light pulse at ExT 4 was on average 0.5 h (SEM 0.3 h). These shifts were not significantly different from zero. They are therefore not in disagreement with the data presented by Albrecht et al. (2001). Since our analysis required individual mice to have clear rhythmic behavior both with and without a light pulse, we could not collect sufficient data for *mPer2*^{Brdm1} mice in experiment 1 due to its frequent arrhythmicity.

When released in DD after entrainment, both *mCry1*$^{-/-}$ and *mCry2*$^{-/-}$ mice did show a considerable average phase advance without light pulses. The average advances in both *mCry1*$^{-/-}$ and *mCry2*$^{-/-}$ mice were larger than those in wild type (1-way ANOVA on ranks; H = 12.0; $df = 2$; $p < 0.005$). After exposure to a light pulse at ExT 20, wild-type mice exhibited a normal and significant delay. Both *mCry1*$^{-/-}$ and *mCry2*$^{-/-}$ mice did show considerable delays after this light

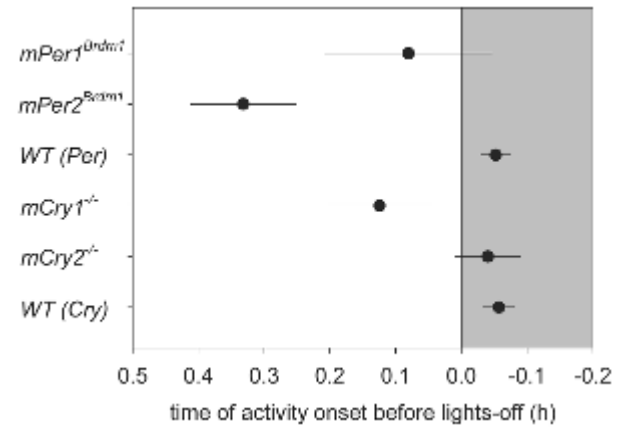

Figure 2. Average position of the onset of activity relative to lights-off (t = 0) during entrainment in LD 12:12 in different genotypes. Error bars indicate 1 SEM.

pulse, larger than that in wild-type mice (1-way ANOVA, $F = 8.3$; $df = 2$; $p = 0.001$), with both *mCry1*$^{-/-}$ and *mCry2*$^{-/-}$ significantly different from wild type (Tukey test, $p < 0.05$ and $p < 0.05$, respectively). Delays in *mCry1*$^{-/-}$ and *mCry2*$^{-/-}$ mice were not statistically distinguishable from each other. When exposed to a light pulse at ExT 4, *mCry1*$^{-/-}$ and *mCry2*$^{-/-}$ mice both showed small average phase advances similar to and not significantly different from those in wild-type mice.

Experiment 2

In total, 139 phase shifts could be measured in DD in wild-type mice (background similar to that of the *mPer* mutants), 79 in *mPer1*^{Brdm1}, 71 in *mPer2*^{Brdm1}, 86 in *mCry1*$^{-/-}$, and 98 in *mCry2*$^{-/-}$ animals. Examples of phase shifts in all genotypes are presented in Figure 5. Phase shifts were excluded if 1) the peak ΔQp value obtained by periodogram analysis (ΔQp is the value for Q at the peak minus the corresponding 0.1% significance value; Sokolove and Bushell, 1978) was smaller than zero for the circadian activity rhythms either before or after a light pulse and if 2) one of the onsets before or after the light pulse was fitted in obvious disagreement with visual inspection of the actogram. This occurred, for instance, when rhythms were ultradian and the positive ΔQp value was because of a subharmonic. It could also be caused by obvious pattern changes. Criterion 2 excluded 19 shifts in *mPer1*^{Brdm1}, 22 shifts in *mPer2*^{Brdm1}, 9 shifts in wild-type, 17 shifts in *mCry1*$^{-/-}$, and 15 shifts in *mCry2*$^{-/-}$ mice.

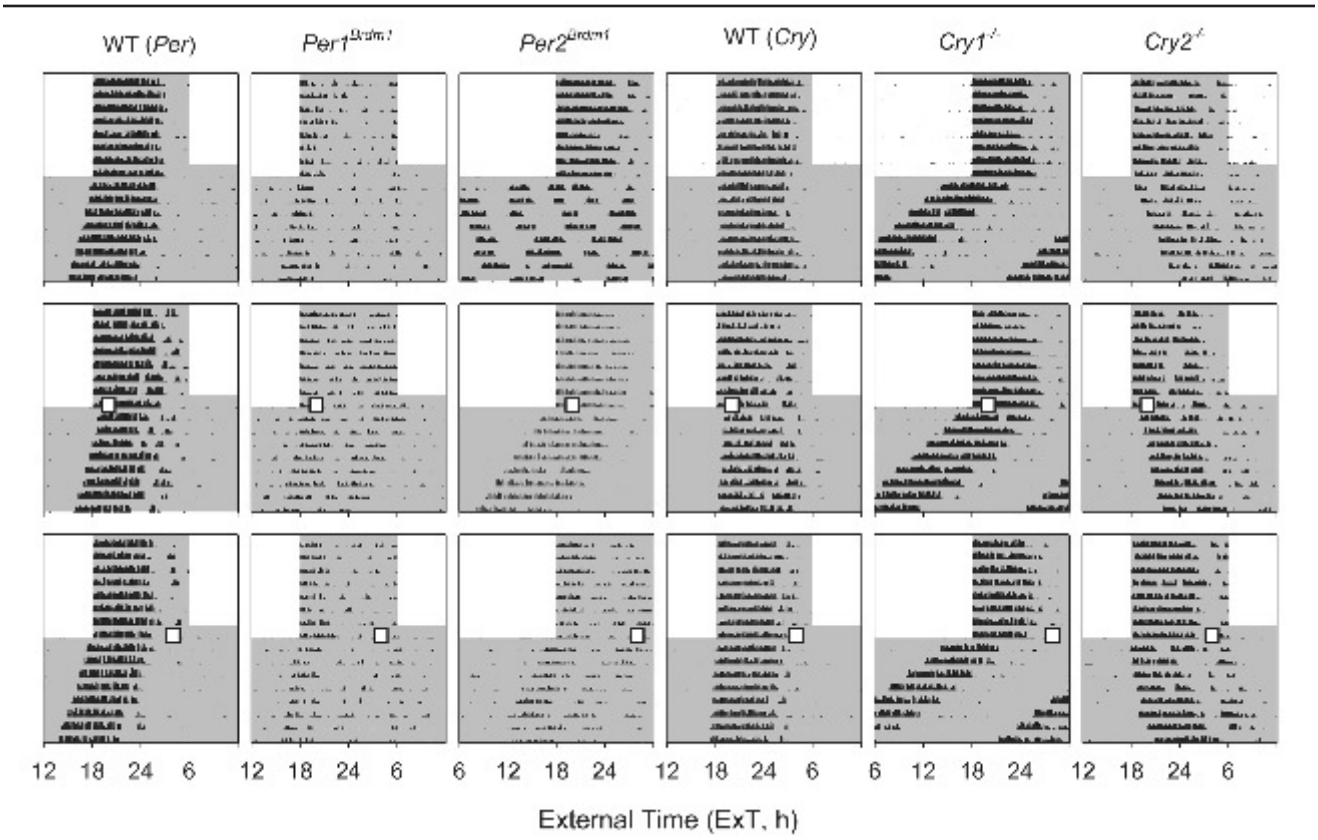

Figure 3. Examples of phase shifts after transition from L:D 12:12 to DD (upper row), with an additional 15′ 480-lux light pulse at ExT 20 (middle row) and at ExT 4 (bottom row) in different genotypes. Gray indicates darkness; light pulses are denoted by open squares.

Rhythmicity in DD

The *mPer2*^{Brdm1} yielded the smallest number of phase shifts, in spite of the extension of the study for this strain. This is again due to the fact that these mice often become arrhythmic in DD (Zheng et al., 1999; Oster et al., 2002). Arrhythmicity often started after a light pulse and could suddenly disappear after another light pulse (Fig. 5, right *mPer2*^{Brdm1} actograms). We analyzed whether the distribution (see Fig. 6A) over the circadian cycle of light pulses that were followed by arrhythmicity differed from random. Responses (binary data) were transformed by the logit link function and analyzed assuming a binomial error distribution on the individual level. In a 2-level model using individual responses, we tested in MLwin 1.1 (Bryk and Raudenbusch, 1993) whether there was heterogeneity with respect to phase. Second-order penalized quasilikelihood estimation was used (Goldstein, 1995); there was no bias toward a particular phase for light pulses followed by arrhythmicity (Walt statistic χ^2 test; $p = 0.32$).

In addition, we analyzed whether light pulses presented to arrhythmic *mPer2*^{Brdm1} mice that became rhythmic immediately after a light pulse ($n = 96$) were concentrated around a particular phase of the new rhythm. Figure 6B demonstrates that these new phases concentrate around InT 13.8. This concentration is decidedly different from a random distribution (χ^2 test; $p < 0.001$). Hence, the pacemaker can apparently spontaneously resume self-sustained DD motion after a light pulse around this circadian phase.

In free run in DD, circadian period lengths measured in between the light pulses were on average 23.5 ± 0.2 h in *mPer1*^{Brdm1} mice, that is, not significantly different from that of wild type (23.5 ± 0.1 h); they were on average 22.7 ± 0.1 h in *mPer2*^{Brdm1} mice (significantly shorter than in wild type; *t* test, $p < 0.001$). The average period was 21.7 ± 0.1 h in *mCry1*$^{-/-}$ mice, significantly shorter than in *mCry2*$^{-/-}$ mice (25.1 ± 0.1 h; *t* test, $p < 0.001$), which is in agreement with the results of van der Horst et al. (1999).

Phase Response Curves

To obtain a quantitative comparison of the PRCs among the genotypes, we first combined data in 12

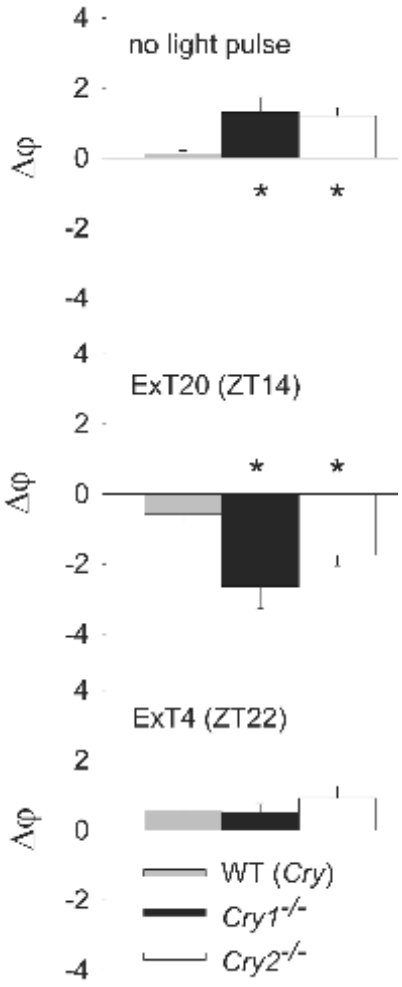

Figure 4. Average phase shifts ± 1 SEM directly after entrainment in *mCry* mutant mice. Phase shifts are calculated based on activity onset and intraindividually corrected for the spontaneous phase shift after transition from L:D 12:12 to DD. Upper graph: no light pulse given. Middle graph: light pulse starting 2 h after last lights-off (external time [ExT] 20). Bottom graph: light pulse starting 10 h after last lights-off (ExT 4). Significant differences from wild type are denoted by asterisks.

bins of 2-circadian-h width, and we calculated the average phase shift per 2-h phase bin for each strain. These data are plotted, along with the SEMs, in Figure 7. The first conclusion from this figure is that all genotypes apparently are capable of both phase advances and phase delays, although the amplitude of the advance section of the PRCs tends to be smaller than that of the delay section. The wild-type PRC corresponds reasonably well with the PRC published for 15′ light pulses in C57Bl6 mice by Daan and Pittendrigh (1976). In the quantitative detail, there are differences.

The PRC for *mPer2*^{Brdm1} appears to show larger advances and smaller delays than do those of the *mPer1*^{Brdm1} mutant and wild-type mice, while delays in *mCry2*$^{-/-}$ on average are smaller than those in *mCry1*$^{-/-}$ mutant mice. We tested the general differences first in ANOVAs on the basis of all 12 bins (Table 1) and then in 3 groups of 4 bins, roughly representing the delay zone (InT 18-2), the advance zone (InT 2-10), and the dead zone (InT 10-18).

In a 2-way ANOVA test, the overall *mPer2*^{Brdm1} PRC is significantly above the *mPer1*^{Brdm1} PRC and the wild-type PRC, and the *mCry*$^{-/-}$ PRC is significantly below the *mCry2*$^{-/-}$ PRC. When the PRCs are tested in 3 sections, a delay section (InT 18-2), an advance section (InT 2-10), and the dead zone section (InT 10-18), significant differences remain present between *mPer2*^{Brdm1} and wild-type mice in the advance and delay sections. The average delay in *mCry2*$^{-/-}$ was significantly higher than the average in *mCry1*$^{-/-}$ mice (Table 1). Significant differences in other sections between different genotypes within strains are absent.

DISCUSSION

mPer Mutants

The results for light pulses presented to *mPer1*^{Brdm1} immediately after entrainment in either the early part or the late part of the subjective night (experiment 1) are not different from the results reported by Albrecht et al. (2001), carried out in the same type II protocol (Aschoff, 1965). Our data for *mPer2*^{Brdm1} mice turned out to be far too few as a consequence of the frequent arrhythmicity in this strain in DD (Zheng et al., 1999; Oster et al., 2002), which often occurred either in the pulse or in the control situation. Thus, we can neither refute nor firmly confirm the observations by Albrecht et al. of suppressed delay responses to light in *mPer2* mutant mice.

The full PRCs of *mPer1*^{Brdm1} and *mPer2*^{Brdm1} show that both genotypes indeed can exhibit both phase advances and phase delays. The *mPer1*^{Brdm1} PRC is in fact virtually on top of the wild-type PRC in the phase delay area, and there are no significant differences between these 2 strains anywhere in the cycle. The difference in response to an ExT 4 light pulse in the data of Albrecht et al. (2001) is not reflected in the full PRCs. This difference is possibly because of different protocols. Differences between LD and DD in the phase relationship between gene expression patterns and locomotor activity, and between individual neurons in the SCN, have been demonstrated by Quintero et al. (2003).

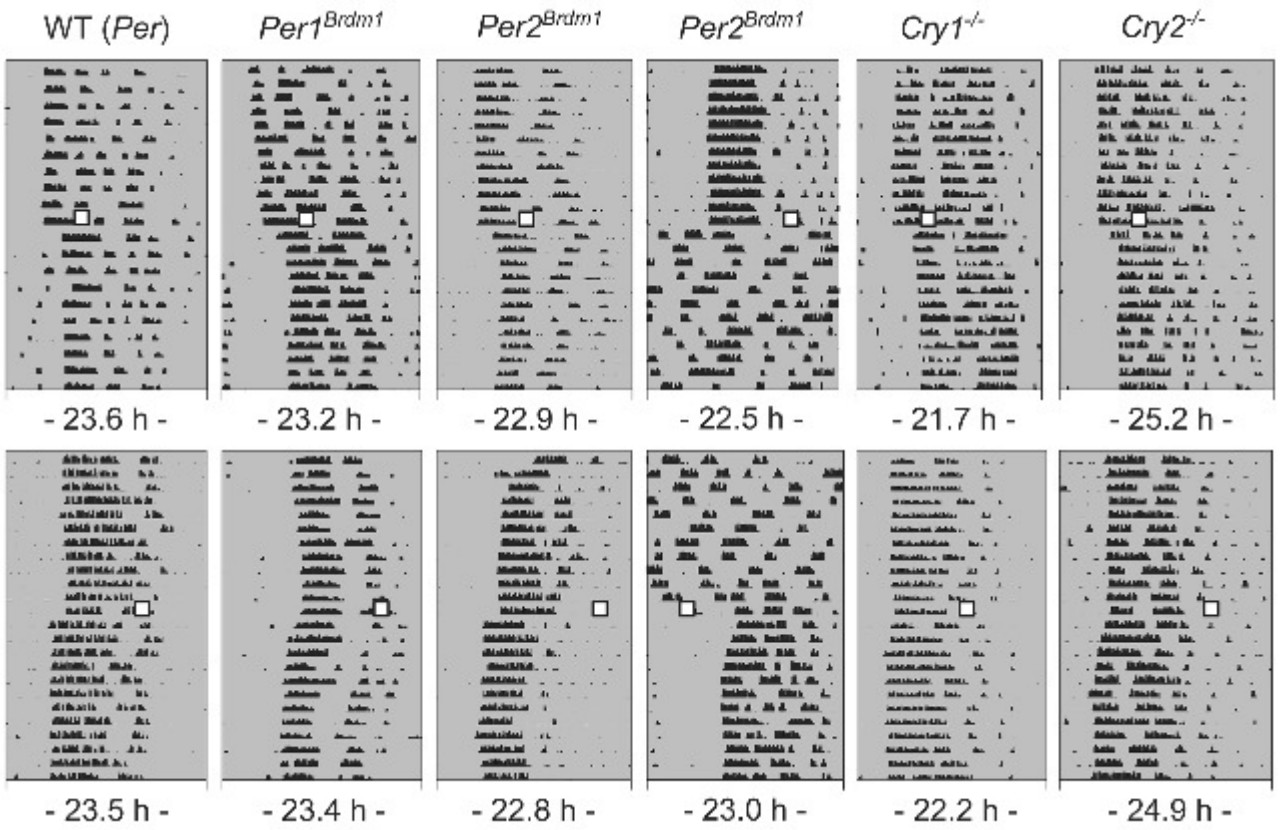

Figure 5. Examples of phase delays (upper row) and phase advances (lower row) in running-wheel behavior in free run in DD in mutant mice, as a result of a 15′ 480-lux light pulse (indicated by open squares). Left *mPer2*^{Brdm1} actograms: arrhythmicity after exposure to a light pulse (upper actogram) and rhythmicity after exposure to a light pulse in a formerly arrhythmic mouse. Activity patterns are plotted at intrinsic τ basis, denoted below each actogram.

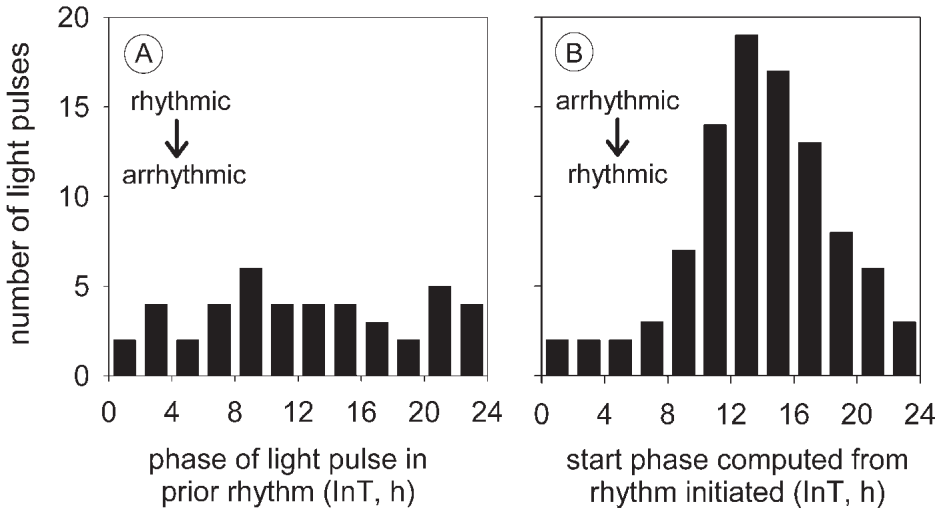

Figure 6. (A) Frequency distribution of circadian phases of light pulses in *mPer2*^{Brdm1} mice that became arrhythmic directly after light exposure. (B) Frequency distribution of circadian phases calculated backwards for the time of the light pulse in *mPer2*^{Brdm1} mice that were arrhythmic before and regained rhythmicity directly after light exposure. InT = internal time.

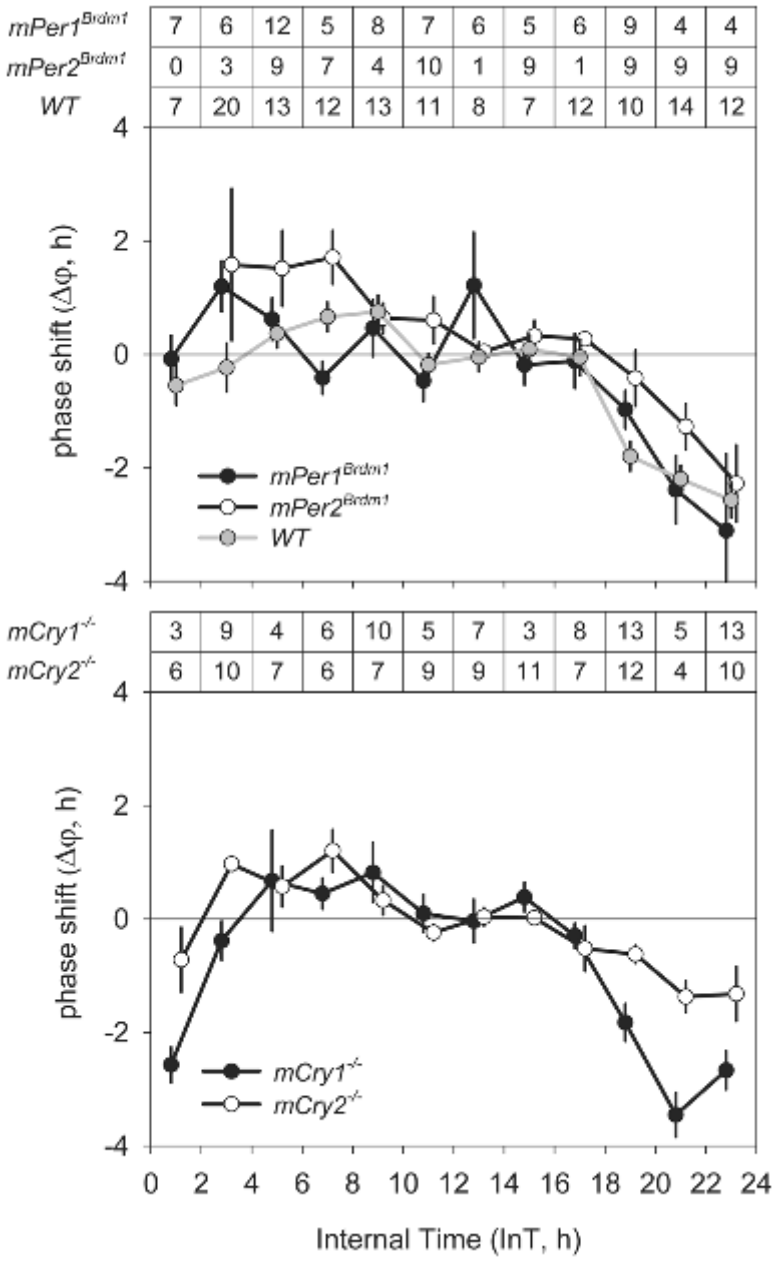

Figure 7. The PRCs of different strains of mice binned in 2-h intervals. Each symbol indicates the mean phase shift of all circadian rhythms in a strain illuminated by a light pulse starting in a 2-h interval of circadian phase (InT 18 = activity onset). Lines indicate 1 SEM on both sides of the mean; numbers above the graphs denote the *n* size for each genotype for each bin.

A difference in phase relationship might still be present in vivo in the 1st cycles after transition from LD to DD, in which light pulses in the type II protocol are administered.

Statistical comparison (Table 1) shows that the PRCs of the 2 *mPer* genotypes differ from the direction of the observations by Albrecht et al. (2001), with more advances in *mPer2*^{Brdm1} mice. The differences in the amplitude of phase shifts from the data of Albrecht et al. may be attributable to the 2 different protocols, even if such protocol differences between Aschoff's type I and type II protocols have not been observed for non-

Table 1. Summary of Phase Shift Data of the Different Strains of Mice in Experiment 2

		Circadian Phase												
		InT 0-24			*InT 18-02 (D)*			*InT 02-10 (A)*			*InT 10-18*			
		Mean	*SEM*	n	*Mean*	*SEM*	n	*Mean*	*SEM*	n	*Mean*	*SEM*	n	*D/A*
	WT(*Per*)	–0.49	0.13	139	–1.90	0.17	43	0.31	0.18	58	–0.06	0.12	38	6.13
	$mPer1^{Brdm1}$	–0.16	0.18	79	–1.30	0.34	24	0.53	0.22	31	0.10	0.28	24	2.47
	$mPer2^{Brdm1}$	0.09	0.23	71	–1.32	0.32	27	1.43	0.31	23	0.44	0.21	21	0.92
ANOVA														
	F	5.0			4.2			4.1			0.5			
	df	2			2			2			2			
	p	<0.01			<0.05			<0.05			*ns*			
Tukey														
$mPer1^{Brdm1}$ – WT(*Per*)		*ns*			*ns*			*ns*			*ns*			
$mPer2^{Brdm1}$ – WT(*Per*)		<0.01			<0.05			<0.05			*ns*			
$mPer1^{Brdm1}$ – $mPer2^{Brdm1}$		<0.05			*ns*			*ns*			*ns*			
	$mCry1^{-/-}$	–0.86	0.18	86	–2.45	0.20	34	0.35	0.24	29	–0.041	0.14	23	7.00
	$mCry2^{-/-}$	–0.13	0.11	98	–0.95	0.19	32	0.78	0.13	30	–0.145	0.09	36	1.23
ANOVA														
	F	16.5			29.5			2.0			1.6			
	df	1			1			1			1			
	p	0.001			<0.001			*ns*			*ns*			

NOTE: The 4 columns present average phase shifts (with SEM and *n*) for the whole circadian cycle (internal time [InT] 0-24), the delay region (D, InT 18-02), the advance region (A, InT 2-10), and the dead zone (InT 10-18). The ANOVA results indicate the statistical significance of variance among strains. In the case of the *mPer* mutant strains, post hoc pairwise comparisons are made by Tukey tests.

photic stimuli to hamsters (Mrosovsky, 1996). A difference between responses in the 2 protocols may be evoked by the light exposure during entrainment in the type II protocol, which may reduce the amplitude of the phase shift. It has long been known that entrainment increases the amplitude of the oscillator ("resonance") and simultaneously reduces the amplitude of the phase shift in response to standard pulses, such that there is slow dark adaptation (Winfree, 1972; Refinetti, 2003).

It is of importance to note that the $mPer2^{Brdm1}$ strain, although showing smaller delay shifts, is surely not insensitive to light. On the contrary, the *mPer2* mutants frequently responded to light pulses with immediate complete arrhythmia, and this effect did not depend on the circadian phase exposed to the light. On the other hand, brief light pulses given to arrhythmic animals were often followed by a spontaneous return of rhythmicity. This is reminiscent of the induction of rhythmicity by longer light pulses described in *clock* mutant mice (Vitaterna et al., 1994; Spoelstra et al., 2002). Apparently, the homozygous *clock* and *per2* mutants share lability of the circadian system in DD and the stabilizing response to single, brief light exposures. In $mPer2^{Brdm1}$, the returning circadian rhythm started from around circadian phase InT 13.8 in the majority of cases. In the *clock* mutant, this has not been investigated.

mCry Mutants

The phase delays observed in $mCry1^{-/-}$ and $mCry2^{-/-}$ after entrainment (experiment 1) were significantly larger than in wild type. This is possibly related to the fact that without a light pulse, both showed a significantly earlier phase than did wild type (Fig. 4). Therefore, the light pulse aimed at ExT 20 may have hit the circadian system at a slightly later phase than it did in wild type. The difference attests to the difficulty in using the type II protocol for PRC studies. The small phase advances following light pulses at ExT 4 were not affected by the phase difference, possibly because around this phase there are less steep changes in the PRC. The genotypic differences in PRCs are more completely reflected in free run (experiment 2). There were particularly distinct differences in the phase delays produced in *mCry* mutant mice in the delay phase between InT 18 and InT 2. This was fully due to strong and highly significant suppression of the phase delay shifts in $mCry2^{-/-}$, at least when compared to $mCry1^{-/-}$ (Table 1).

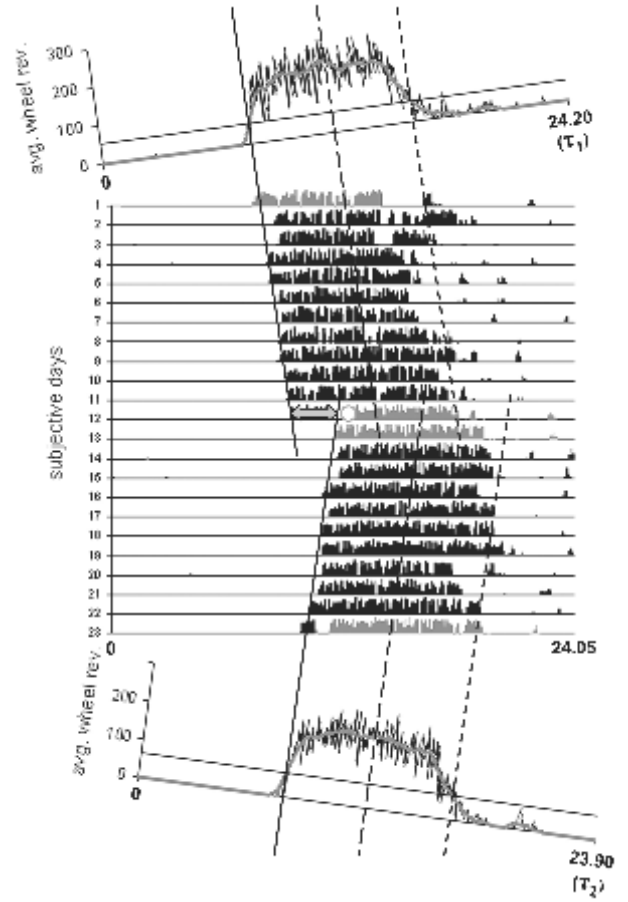

Figure 8. The automatic phase shift calculation method. Actogram of running-wheel activity data plotted on basis of the average of τ before (τ_1) and τ after (τ_2) the light pulse. Data omitted to avoid effects of transient tau values are plotted in gray. The open circle indicates the light pulse. Upper and lower graph: average activity pattern before and after the light pulse, respectively. Straight lines denote onset of activity, medium dashed lines denote circular center of gravity, and dashed lines denote offset of activity. The gray arrow indicates the actual phase shift.

CONCLUSION

Taken together, phase responses are more positive and/or less negative in *mPer2* than in *mPer1* mutants, and the same is true of *mCry2* compared to *mCry1* mutants. This means that *mPer2* and *mCry2* mutant circadian systems are more accelerated and/or less decelerated by light than are *mPer1* and *mCry1* mutants, respectively. At cursory inspection, these results seem to qualitatively fit with the predictions generated from the molecular 2-component model for rodent circadian systems (Daan et al., 2001). However, that model made more specific predictions concerning the difference from wild type. A difference from wild type has so far been found only in the *mPer2* mutant. This mutant has other dominant characteristics, such as the tendency to become arrhythmic in DD, which may be related to the deviant PRC. Therefore, we have to be careful in interpreting the results as supporting the 2-component hypothesis. More work is needed employing different protocols to arrive at a firm conclusion in this respect. Be this as it may, the data clearly demonstrate that all mutant strains retain both advancing and delaying responses. All can be expected to entrain to both longer and shorter Ts, as observed by Bae and Weaver (2003) for *Per* mutants, although the ranges of entrainment may show only partial overlap.

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APPENDIX

Assessing phase shifts from automatically collected data in a standardized, automated way has always been a complicated issue. To calculate accurate phase shifts in a standardized way, proper phase markers are of utmost importance. Usually, phase markers are detected for each individual cycle in an entrained or free-running rhythm. In free run, the circadian system reverts to its original steady state after the light pulse. Phase shifts are assessed by drawing lines (regression or eye fitted) through consecutive phase markers for free runs before and after a light pulse. Although interobserver consistency in eye-fitting procedures (Pittendrigh and Daan, 1976a) has often been considered to supp

ort the precision of the procedure, this does not mean that the procedure is also accurate. To obtain an objective measure of phase shift, we developed an automatic means of phase calculation in which phase markers before and after a light pulse are based on all circadian cycles. The software that performs these calculations will be made available by the first author on request. The calculations proceed in 4 steps:

Step 1. The program first calculates the period length of the activity rhythm before (τ_1) and after the light pulse (τ_2). This is done by running a periodogram analysis (Sokolove and Bushell, 1978) on all activity data starting from 10 days before the light pulse until the time the light pulse starts, and again on all activity data

APPENDIX (continued)

from 2 days until 12 days after the light pulse. Data from the first 2 days after the light pulse are omitted to exclude transient cycles. All calculations described are based on these 2 time windows.

Step 2. The circadian activity pattern before and after the light pulse is averaged over τ_1 and τ_2 , respectively. This is done by averaging the activity values with a distance equal to a multiple of τ in τ/(sample interval) tallies, the 1st tally starting with the 1st sample of the data range (see Fig. 8).

Step 3. The program calculates the (circular) center of gravity in each of the 2 average activity patterns. It then searches the 1st time interval in which the activity count exceeds the average value of all tallies, starting $0.5 * \tau$ prior to the center point of gravity. The offset of activity is detected in the same way but by going backwards in time starting $0.5 * \tau$ after the center point of gravity. The program can be instructed to carry out this procedure either on the raw data or on running means over adjustable time windows. In the analyses in this article, we always used 1-h running means.

Step 4. The detected phase markers are projected on the actogram (see Fig. 8). The straight lines denote onsets, the medium dashed lines denote the center points of gravity, and the dashed lines denote the offsets of activity. The actual phase shift is defined as the difference between the phase angle at the time of the light pulse calculated on the basis of the onset of activity before (φ_1) and after the light pulse (φ_2). The internal times of φ_1 and φ_2 are calculated as follows:

$$\varphi_1 = (((t - ons_1) \bmod \tau_1) * 24/\tau_1 + 18) \bmod 24 hours (InT)$$

$$\varphi_2 = (((t - ons_2) \bmod \tau_2) * 24/\tau_2 + 18) \bmod 24 hours (InT),$$

where tLP = time of exposure to the light pulse; ons_1 = moment of onset derived from the average activity pattern before the light pulse; ons_2 = moment of onset derived from the average activity pattern after the light pulse.

With these 2 phase angles of the light pulse, the actual phase shift is calculated:

$$\Delta\varphi = \varphi_2 - \varphi_1 \text{ (circadian hours)}.$$

REFERENCES

Albrecht U, Zheng B, Larkin DW, Sun ZS, and Lee CC (2001) *mPer1* and *mPer2* are essential for normal resetting of the circadian clock. J Biol Rhythms 16:100-104.

Aschoff J (1965) Response curves in circadian periodicity. In *Circadian Clocks*, Aschoff J, ed, pp 95-111, North-Holland, Amsterdam.

Bae K and Weaver DR (2003) Light-induced phase shifts in mice lacking mPER1 or mPER2. J Biol Rhythms 18:123-133.

Bryk AS and Raudenbusch SW (1993) *Hierarchical Linear Models: Application and Data Analysis Method*, Sage, Newbury Park, CA.

Daan S, Albrecht U, van der Horst GTJ, Roenneberg T, Roenneberg T, Wehr TA, and Schwarz WJ (2001) Assembling a clock for all seasons: Are there M and E oscillators in the genes? J Biol Rhythms 16:105-116.

Daan S, Merrow M, and Roenneberg T (2002) External time—internal time. J Biol Rhythms 17:107-109.

Daan S and Oklejewicz M (2003) The precision of circadian clocks: Assessment and analysis in Syrian hamsters. Chronobiol Int 20:209-221.

Daan S and Pittendrigh CS (1976) A functional analysis of circadian pacemakers in nocturnal rodents II: The variability of phase response curves. J Comp Physiol 106:253-266.

Goldstein H (1995) *Multilevel Statistical Models*, Edward Arnold, London.

Ilnerová H and Vanecek J (1982) Two-oscillator structure of the pacemaker controlling the circadian rhythm of N-acetyltransferase in rat pineal gland. J Comp Physiol [A] 145:539-548.

Jagota A, de la Iglesia HO, and Schwarz WJ (2000) Morning and evening circadian oscillations in the suprachiasmatic nucleus *in vitro*. Nat Neurosci 3:372-376.

Mrosovsky N (1996) Methods of measuring phase shifts: Why I continue to use an Aschoff type II procedure despite the skepticism of referees. Chronobiol Int 13:387-392.

Oster H, Yasui A, van der Horst GTJ, and Albrecht U (2002) Disruption of *mCry2* restores circadian rhythmicity in *mPer2* mutant mice. Genes Dev 16:2633-2638.

Pittendrigh CS and Daan S (1976a) A functional analysis of circadian pacemakers in nocturnal rodents, I: The stability and lability of spontaneous frequency. J Comp Physiol 106:223-252.

Pittendrigh CS and Daan S (1976b) A functional analysis of circadian pacemakers in nocturnal rodents, V. Pacemaker structure: A clock for all seasons. J Comp Physiol 106:333-355.

Quintero JE, Kuhlman SJ, and McMahon DG (2003) The biological clock nucleus: A multiphasic oscillator network regulated by light. J Neurosci 23:8070-8076.

Refinetti R (2003) Effects of prolonged exposure of darkness on circadian photic responsiveness in the mouse. Chronobiol Int 20:417-440.

Sokolove PG and Bushell WN (1978) The chi square periodogram: Its utility for analysis of circadian rhythms. J Theor Biol 72:131-160.

Spoelstra K, Oklejewicz M, and Daan S (2002) Restoration of self-sustained circadian rhythmicity by the mutant *Clock* allele in mice in constant illumination. J Biol Rhythms 17:520-525.

van der Horst GTJ, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M, de Wit J, Verkerk A, Eker APM, van Leenen D, et al. (1999) Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature 398:627-630.

Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, McDonald JD, Dove WF, Pinto LH, Turek FW, and

Takahashi JS (1994) Mutagenesis and mapping of a mouse gene, *Clock*, essential for circadian behavior. Science 264:719-725.

Wehr TA (1997) Melatonin and seasonal rhythms. J Biol Rhythms 12:517-526.

Winfree AT (1972) Slow dark-adaptation in *Drosophila*'s circadian clock. J Comp Physiol 77:418-434.

Zheng B, Larkin DW, Albrecht U, Sun ZS, Sage M, Eichele G, Lee CC, and Bradley A (1999) The *mPer2* gene encodes a functional component of the mammalian circadian clock. Nature 400:169-173.