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Phase and Period Responses of the Circadian System of Mice (*Mus musculus*) to Light Stimuli of Different Duration

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Abstract To understand entrainment of circadian systems to different photoperiods in nature, it is important to know the effects of single light pulses of different durations on the free-running system. The authors studied the phase and period responses of laboratory mice (C57BL6J//OlaHsd) to single light pulses of 7 different durations (1, 3, 4, 6, 9, 12, and 18 h) given once per 11 days in otherwise constant darkness. Light-pulse duration affected both amplitude and shape of the phase response curve. Nine-hour light pulses yielded the maximal amplitude PRC. As in other systems, the circadian period slightly lengthened following delays and shortened following advances. The authors aimed to understand how different parts of the light signal contribute to the eventual phase shift. When PRCs were plotted using the onset, midpoint, and end of the pulse as a phase reference, they corresponded best with each other when using the mid-pulse. Using a simple phase-only model, the authors explored the possibility that light affects oscillator velocity strongly in the 1st hour and at reduced strength in later hours of the pulse due to photoreceptor adaptation. They fitted models based on the 1-h PRC to the data for all light pulses. The best overall correspondence between PRCs was obtained when the effect of light during all hours after the first was reduced by a factor of 0.22 relative to the 1st hour. For the predicted PRCs, the light action centered on average at 38% of the light pulse. This is close to the reference phase yielding best correspondence at 36% of the pulses. The result is thus compatible with an initial major contribution of the onset of the light pulse followed by a reduced effect of light responsible for the differences between PRCs for different duration pulses. The authors suggest that the mid-pulse is a better phase reference than lights-on to plot and compare PRCs of different light-pulse durations.

Key words circadian clock, phase resetting, phase response curve, period response curve, light pulse

In nature, virtually all circadian rhythms assume the 24.0-h period of the solar day-night cycle. This is due to the entrainment of the endogenous oscillators to the external light-dark cycle, the dominant zeitgeber for the majority of organisms. The process of entrainment is based on differential phase and period responses of the circadian systems to light depending on the phase at which the stimulus is applied. The

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phase dependence is described empirically by the phase response curve plotting the phase shifts induced by a single standard stimulus as a function of the circadian phase at which it is applied (Bruce and Pittendrigh, 1958). The shape of the PRC for light pulses is qualitatively similar for all circadian systems studied, with phase delays elicited in the early subjective night and phase advances in the late subjective night. Quantitatively, the PRC is determined by properties both of the system studied and of the probing light pulse. Essential properties of the light signal are its duration, intensity, and spectral composition. In this article, we focus on the effect of pulse duration on phase shifts, as well as on period responses.

The relationship between phase shifts induced by light pulses of varying duration and intensity is of particular importance for our understanding of the mechanisms of entrainment. The emphasis in chronobiology has been on instantaneous, discrete, daily phase shifts in response to brief stimuli. In nature, brief pulses rarely occur. Pulse PRCs often fail to predict the behavior under artificial (Pittendrigh and Daan, 1976b) or natural (Hut et al., 1999) entrainment conditions. This is partly because exposure to a zeitgeber itself affects the period of the circadian system (τ) and the PRC, and partly because the action of long light pulses such as the daily photoperiod may not be readily deducible from that of short pulses.

The period of circadian systems is subject to systematic induced variations. These are known as *after-effects* (Pittendrigh, 1960). Changes in τ following single light pulses usually are in the same direction as the phase shifts: reductions of τ are often associated with phase advances elicited by the same pulse, increases of τ with phase delays (Pittendrigh and Daan, 1976a). Besides instantaneous phase shifts, there are thus subtle changes in the velocity of circadian systems in response to light and to the repetitive action of the zeitgeber. Disentangling the contributions of instantaneous phase shifts and of parametric effects on angular velocity has been notoriously difficult (Beersma et al., 1999a, 1999b).

In the 1960s and 1970s, there were 2 dominant approaches to the problem of entrainment. Colin Pittendrigh emphasized the nonparametric, instantaneous phase-shifting action of light, while Jürgen Aschoff employed models entailing the parametric changes in angular velocity due to prolonged light exposure (Daan, 2000). Pittendrigh (e.g., 1960, 1972) argued that transitions from light to darkness and from darkness to light are the crucial stimuli for the entrainment of the circadian system. He further suggested that the entrainment action of a complete photoperiod may be largely explained by instantaneous action of the light transitions at the beginning and end of the photoperiod (Pittendrigh and Minis, 1964). Pittendrigh's approach was successful in predicting the steady-state entrainment in *Drosophila* under unusual light-dark cycles from the response to single, brief light pulses (Pittendrigh, 1960). Possibly as a consequence, the bulk of later research has focused on the action of brief light pulses (e.g., DeCoursey, 1960; Pittendrigh and Daan, 1976b; Johnson, 1991). Aschoff and Wever (Aschoff, 1964; Wever, 1966) established the parametric model of entrainment emphasizing continuous changes in the angular velocity of oscillators under the influence of light. They assumed that part of the endogenous cycle is accelerated and another part is decelerated by light. Aschoff (1963) was always well aware of the possibility of a combination of parametric and nonparametric effects in entrainment, and indeed, in Wever's (1966) mathematical model, both light intensity and its time derivatives exert their influence. Regardless of the parametric and nonparametric terminology, it is important for the understanding of entrainment to know how different parts of long light pulses contribute to both period and phase responses.

Studies addressing the duration of single light pulses have focused on brief pulses rather than on the long durations that occur in natural photoperiods. Winfree (1970) varied duration and intensity of light pulses in his search for the singularity of circadian clocks underlying the *Drosophila* pupal eclosion rhythm. The intensity-duration reciprocity was also the theme of a seminal study on hamster phase shifts by Nelson and Takahashi (1991). The durations investigated in both studies were restricted to the minute range. One study evaluated the phase responses for a series of light pulses from 1 to 20 h in the blowfly *Sarcophaga argyrostoma* (Saunders, 1978). These blowfly data are difficult to interpret owing to the discontinuity between type-1 PRCs for the short pulses and type-0 for the long pulses.

We embarked on an extensive analysis in individual animals, with solely weak phase resetting (type-1 PRC). We chose to do this in mice in view of the important role of mice in recent progress in understanding the molecular basis of circadian timing and entrainment. Light-pulse PRCs have been measured in house mice before (Daan and Pittendrigh, 1976a; Spoelstra et al., 2004), but not with pulses longer than 15 min. In the present study, we applied 7 different light-pulse durations to

construct phase response curves. We aimed first to establish the light reference phase that leads to maximal correspondence between responses to pulses of different duration. This provides information on how different parts of the pulse contribute to the phase-shift response. For instance, if all shifting is elicited by the 1st hour of light, PRCs should line up when plotted relative to the circadian phase at the onset of the pulse. Following this principle, the PRCs can be exploited to estimate the relative effects of onset, end, as well as intermediate light. We further aimed to evaluate after-effects of single light stimuli on τ, as well as the relationship between phase and period responses when different durations of light pulses are given.

MATERIALS AND METHODS

Animals and Maintenance

Ninety-six male wild-type C57BL/6JOlaHsd mice (*Mus musculus*) were used. This strain is characterized by being deficient in melatonin production (Ebihara et al., 1986). They were obtained from Harlan (Horst, the Netherlands) when circa 60 days old. They were housed individually in lucite cages (25 × 25 × 40 cm) in a sound-attenuated and climatized room (temperature 23 ± 1 °C) with food (Hope Farms standard rodent pellets, Arie Block, Woerden, the Netherlands) and water ad libitum. Spontaneous locomotor activity was recorded with running wheels (Ø 14 cm) connected to an event recording system storing numbers of wheel revolutions in 2-min intervals. The room used for the experiments is equipped with 24 separate light-tight compartments, each with a computer-controlled variable light intensity provision without changes in wavelength or in ambient temperature. In each compartment, 4 cages were placed each with 1 mouse. All cages were roughly equal distance (70 cm) to the light source.

Experimental Protocol

The mice were initially entrained for 2 weeks in LD (light:dark) 12:12, such that the experimental treatment started with all animals in the same phase. All mice then were released in constant darkness and exposed to a light pulse once every 11 days. Light pulses were applied in 12 compartments at 12 different time points (local time), with 2-h intervals. The 1st compartment received the light pulse starting at 0000 h local time, the next compartment starting at 0200 h, and so on. In this manner, we could present a light stimulus of a specific duration to 48 animals in 12 compartments, while simultaneously administering a stimulus of a different duration to 48 other mice. In total, we tested 7 light-pulse durations: 1, 3, 4, 6, 9, 12, and 18 h. To avoid age contamination in the results, we alternated long and short light pulses. All light pulses were provided by white fluorescent tubes (Osram L58W/31, Philips, Hagemeyer, Netherland B. V. Oostwold, the Netherlands) and had an intensity of circa 100 lx, equivalent to circa 145 mW/m^2 at the cage floor level. Because of the background DD conditions, individual rhythms gradually lost synchrony as a consequence of the slight differences in the period of the free-running rhythms. This resulted in a more or less random distribution of light pulses over the circadian cycle. When there were intervals in the cycle exceeding 3 h without data points, we repeated the procedure to obtain more data points and to fill these gaps. The whole experiment took 253 days, such that the mice were 313 days old at the end of the experiment.

Determination of Phase Shifts ($\Delta\varphi$) and Period Changes ($\Delta\tau$)

Phase shifts were calculated by determining the phase (φ_1) in the cycle at which the light pulse occurred by forward extrapolation from the rhythm before the light pulse and the phase (φ_2) of the same event calculated by backward extrapolation from the rhythm after the light pulse, as described by Spoelstra et al. (2004). These phases were derived from τ_1 and τ_2, the periods before and after the pulse, quantified by periodogram analysis over 10 days of activity, excluding the first 2 days after the light pulse to allow for transients to fade away. Activity onset in these profiles was determined on the basis of the average activity profile over the 10 days, and defined as the 1st time, going forward from the nadir (center of gravity minus half-τ), that the profile exceeded the overall mean activity, and was set at InT (internal time) 18 (which corresponds to CT 12; see Daan et al., 2002). The phase shift calculated is simply $\Delta\varphi = (\varphi_2 - \varphi_1)$. Phase shifts were excluded if one of the onsets before or after the light pulse was fitted in obvious disagreement with the visual inspection of the actogram; that is, an unusual burst of activity in the middle of the subjective day, due to feeding or cleaning of the cages, was taken as the onset of activity

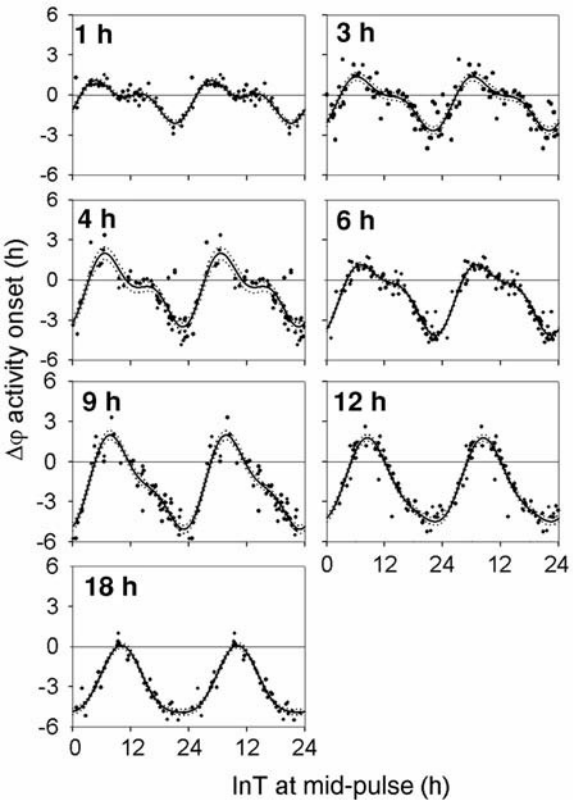

Figure 1. Double-plot PRCs constructed for mouse *Mus musculus*, for light pulses of different durations as indicated in the left upper corner of each graph. Phase shifts calculated at the activity onsets and indicated in hours are plotted against the internal time (InT) at mid-pulse (Internal Time 18 = activity onset). Solid lines indicate 2 harmonics Fourier-fitted curves, and dotted lines show SE of the Fourier-fitted curve.

by the program. This happened in 61 of 422 actograms (14%).

Period changes were defined as $\Delta\tau = \tau_1 - \tau_2$. An increase in τ (deceleration) thus results in a negative value of $\Delta\tau$. A decrease in τ (acceleration) results in a positive $\Delta\tau$.

RESULTS

Phase Response Curves

Up to 361 phase shifts and τ changes were measured (43 after a 1-h light pulse; 56 after 3 h; 52 after 4 h; 68 after 6 h; 50 after 9 h; 55 after 12 h; and 37 after 18 h). For each pulse duration, phase shifts were calculated on the basis of the daily onsets of activity (see Methods and Spoelstra et al., 2004). Phase response curves were obtained by plotting those shifts (in circadian hours) as a function of the phase (InT in hours) the rhythm would have reached at the time of the middle of the light pulse. For each of the light-pulse durations, a curve was fitted to the data based on harmonic regression analysis on nonequidistant data. Two harmonics were taken into account. The standard error range around the curve was also calculated and plotted. Figure 1 provides an overview of the raw data and the harmonic fits. The figure demonstrates that over the entire range of pulse durations from 1 to 18 h, the PRCs remain of type-1 (Winfree, 1970), that is, weak phase resetting.

The duration of the light pulse does affect both the amplitude and the shape of the PRC. The amplitude, defined as half the distance between minimum and maximum values of the harmonic regression, varies from 1.59 h (1-h pulses) to 3.52 h (9-h pulses) (Table 1). This is mainly due to an increase in the maximal delays generated. With increasing pulse duration, the shape of the PRC changes in several respects. First, the dead zone gradually disappears with longer pulses: it is circa 5 h wide in the 1-h, 3-h, and 4-h PRCs, and it starts to disappear in the 6-h PRC. It is lost from the 9-h PRC onward. While the dead zone disappears, there is an increase in the width of the delay zone of the PRC from circa 11 h in the 1-h, 3-h, 4-h, and 6-h PRCs until circa 16 h in the 9-h PRC and circa 18 h in the 12-h PRC. The advance zone of the curves

Table 1. Summary Data Where It Is Shown for Each Light-Pulse Duration the Number of Shifts That Were Measured and Used for Analysis

Pulse Duration (h)	*Number of Shifts*	*Maximum Advance (h)*	*Maximum Delay (h)*	*PRC Amplitude (h)*
1	43	1.10	–2.09	1.59
3	56	1.38	–2.65	2.01
4	52	2.02	–3.49	2.75
6	68	1.15	–4.14	2.64
9	50	1.98	–5.06	3.52
12	55	1.78	–4.48	3.13
18	37	0.09	–4.91	2.50

NOTE: Furthermore, for each phase response curve, using the harmonic regression fitted curves, the maximum delay and advance (in hours) and the correspondent amplitude are detailed.

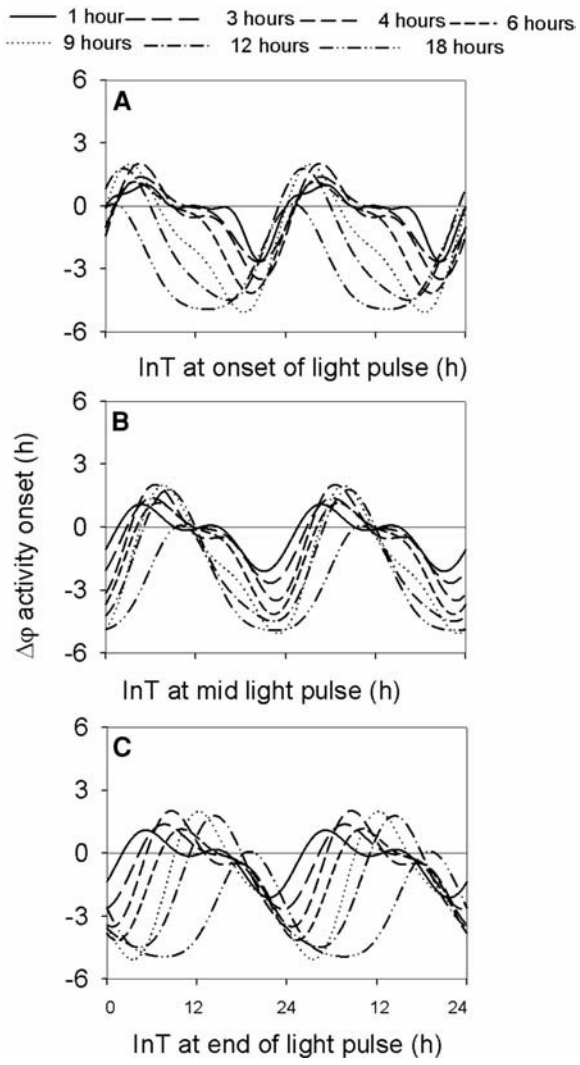

Figure 2. Fourier-fitted curves of the phase response curves for light pulses of different duration plotted relative to (A) internal time at onset of the light pulse, (B) internal time at mid-pulse, and (C) internal time (InT) at offset of light pulse. In plot A, the curves superimpose in the slope that drives to the advancing part of the PRC, whereas it does very poorly in the slope that drives to the delaying part of the PRCs. In plot C, the contrary happens: the curves superimpose in the slope that drives to the delaying part but not to the advancing part of the PRCs. In plot B, the curves superimpose the best.

is about 8 h wide in all PRCs except in the 18-h PRC, where it is virtually absent. Second, the average levels of the PRCs tend to be lower with longer pulses: while phase advances and phase delays are more or less balanced for the short-duration pulse PRCs, with longer pulse durations, the advances are reduced and the delays increase.

The range of phases at which the delays and advances are produced is also affected by the light-pulse duration. The PRCs in Figure 1 were plotted relative to the phase at the midpoint of the pulse. Technically, the choice of the midpoint of the pulse is arbitrary: as long as we know which reference point is used, the plots can be interpreted, no matter how they are plotted. With respect to the underlying mechanisms, it is worthwhile to go through a series of reference points and investigate which reference point generates the closest correspondence between the PRCs. For that purpose, we plotted in Figure 2 the harmonic regressions for all the different light-pulse duration PRCs relative to the phase of the rhythm at the onset (Fig. 2A), the midpoint (Fig. 2B), and the end of the light pulse (Fig. 2C). When the PRCs are aligned relative to the onset of the light pulse, the curves superimpose, in a short-phase angle interval, in the upward slope at the start of the advance part of the PRCs. They show poor correspondence in the downward slope at the start of the delay part of the PRCs. When the curves are plotted as a function of the phase at the end of the light pulse, the opposite happens: the curves superimpose, in a short-phase angle interval, in the downward but not the upward slope. The best overall correspondence is reached when the curves are plotted relative to the midpoint of the pulses.

This conclusion is based on mere visual inspection. We took a more quantitative approach and calculated the average correlation coefficient among the fitted PRCs for a fine-grid series of 21 combinations of the 7 harmonic regressions, 1 per light-pulse duration. We did this not only for the plots against onset (0% of the light pulse), midpoint (50%), and end (100%) but also for intermediate choices of phase reference points. The correlation coefficients were averaged and plotted in Figure 3 in steps of 10%. The maximal correlation (mean coefficient, 0.90) was found for a phase reference at 36% of the duration of the light pulse. While this maximum value cannot be statistically distinguished from a phase reference halfway into the light pulse, it is clear that these references yield much better correspondence than those at the onset or the offset of the pulse.

Period Response Curves

Throughout the experiment, the average τ was shorter than 24 h. Period or τ response curves were obtained by plotting the change of period in response to light pulses calculated at the InT using the midpoint of the light pulse as a phase reference (Fig. 4).

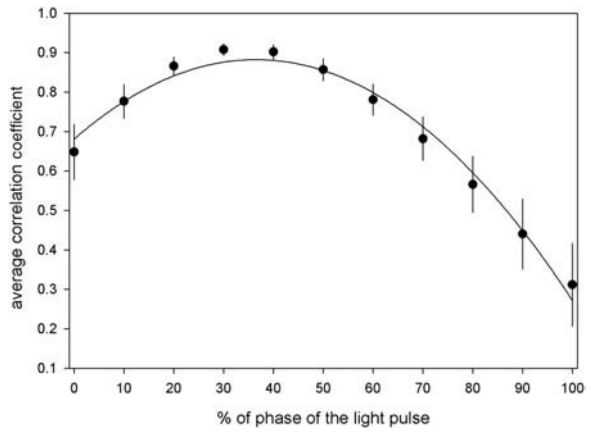

Figure 3. Average (± SEM) correlation coefficients of the fitted phase shifts for all possible combinations of the Fourier-fitted curves of the PRCs for the different light-pulse durations. This was done between 0% of phase of the light pulse (onset of the light pulse) and 100% of phase of the light pulse (offset of the light pulse) in steps of 10%. A quadratic regression curve has been fitted. The maximum correlation lies between 30% and 40% of the duration of the light pulse.

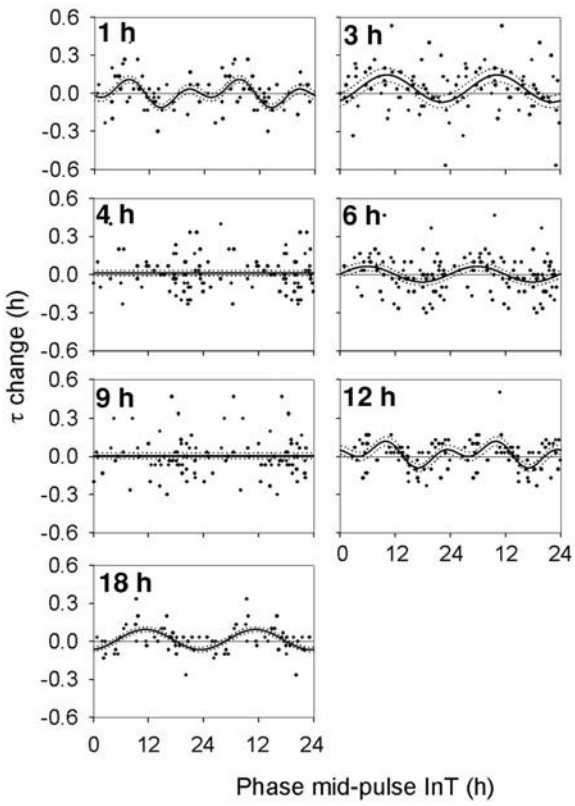

Figure 4. Double plot of τ response curves constructed for mouse *Mus musculus*, for light pulses of different durations as indicated in the left upper corner of each graph. Period changes calculated at the activity onsets and indicated in hours are plotted against the internal time (InT) at mid-pulse (Internal Time 18 = activity onset). Solid lines indicate Fourier-fitted curves, and dotted lines show SE of the Fourier-fitted curve.

Each τRC was fitted by harmonic regression. The number of harmonics included depended on their significance in explaining part of the variance in the data. Only harmonics with significant ($p < 0.05$) explanatory power were included. Following this procedure, 0 harmonics were included for light pulses of 4- and 9-h duration, meaning that there was no significant circadian variation in Δτ. One harmonic was included for 3-h, 6-h, and 18-h light pulses. Two harmonics were included for 1-h and 12-h light pulses. The plots in Figure 4 include the standard error of the means.

We have further evaluated the association of Δτ with Δφ. The phase shifts were significantly positively correlated with the period changes, but the explained variance is rather marginal ($r = 0.13$; $n = 361$; $p < 0.01$). The association is primarily due to significant positive correlations for the 3-h ($r = 0.26$; $n = 56$; $p < 0.05$) and 18-h light pulses ($r = 0.49$; $n = 37$; $p < 0.01$). The other pulses yielded no significant results when analyzed separately.

DISCUSSION

The PRCs (Fig. 1) and τRCs (Fig. 4) demonstrate clearly that both short and long pulses elicit responses in circadian phase as well as period in mice. It is remarkable that the duration of the light pulse is of little influence on the phase shifts and period changes. It is known from at least 2 insect species that very brief light pulses produce type-1 PRCs (weak phase resetting), while pulses longer than 1 min (*Drosophila pseudoobscura*; Winfree, 1970) or longer than 3 h (*Sarcophaga argyrostoma;* Saunders, 1978) generate strong phase resetting (type-0). In mammals, there is only a single study on the effect of long light-pulse durations, in *Rattus exulans* (Gander and Lewis, 1983). This study claims weak resetting by 4-h light pulses and strong resetting by 8-h and 16-h light pulses, but the data leave room for the alternative interpretation of consistently strong, type-0 resetting. In our mice, increasing duration of the light pulse did not result in a transition from weak to strong phase resetting. PRC remained of type-1 for all light-pulse durations tested at 100 lx. Below we discuss 1st period resetting, then phase resetting.

Period Response Curves

Light not only elicits phase shifts but also τ changes that can likewise be plotted in a τ response curve. In mice, like in other nocturnal animals, changes in τ induced by single light pulses seem to be smaller than in diurnal mammals (Beersma et al., 1999a), even though they were originally reported for nocturnal rodents (Pittendrigh and Daan, 1976a). Nonetheless, phase-dependent τ changes in our study were observed for most of the light-pulse durations (Fig. 4). In most cases, Δτ appears to peak between InT 6 and 12, that is, with the midpoint of the light pulse in the early part of the subjective day, and to have a trough between InT 18 and 24, in the early subjective night. The same is true for the PRCs (Fig. 1). Thus, τ lengthens in response to light at the circadian phase, where delay shifts are maximal, and shortens at the circadian phase, where phase shifts are minimal. This association has been observed in other mammals before (Pittendrigh and Daan, 1976a; Kramm and Kramm, 1980; Gerkema et al., 1993; Beersma et al., 1999a; Weinert and Kompauerova, 1998). It demonstrates that the single instantaneous phase shift is part of a more long-term response, which has a clear function in eventually making the system run on a period more closely matching that of its zeitgeber, so that less corrective resetting is required day after day (Beersma et al., 1999a). However, the average τ responses in mice are small, and the variance around the average τRC relatively large. The τ responses are not differentiated pronouncedly between different pulse durations and hence do not elicit much further speculation at the moment. It is important, however, that the positive correlation between Δτ and Δφ is documented in this large database for mice.

Comparison of Phase Response Curves

PRCs varied between light-pulse durations quantitatively if not qualitatively. The maximum delays increased with increasing light pulses up to 9 h and remained stable for pulses from 9 h to 18 h long (Table 1). This effect may be explained by the interpretation already given by Pittendrigh (1960) concerning *Drosophila* data: At maximal delay, short pulses only hit part of the circadian interval where delays are generated. Saturation is reached at 6-h pulses, which generate the largest delays, and apparently can cover the whole delay zone. Longer pulses (9-12 h) hit the delay phase plus the dead zone, while the longest pulses (18 h) hit these as well as a little of the advance phase. Therefore, the response of the system to very long duration light pulses becomes a combination of morning advance and the evening delay responses. This reasoning also explains the disappearance and the complete loss of the dead zone in the PRCs by light pulses of 6 h and longer, respectively. If only long light pulses (from 9-h duration onward) would have been used in this study, we might have concluded that the dead zone is nonexistent in *Mus musculus* as was concluded for 5-h light pulses in humans (Jewett et al., 1997). It might be interesting to assess with short light pulses whether also the human circadian system contains a light-insensitive zone.

The maximum advance remained about equal (circa 2 h) over the range of light pulses from 1 until 12 h and then dropped to virtually zero for light pulses of 18 h. This pattern is expected because the advance zone and dead zone for 1-h light pulses last about 8 and 5 h, respectively, and therefore longer light pulses would not cause much of an increase as long as the extension fills the dead zone, until about 13 h. When a longer pulse (e.g., of 18 h) hits the advance part, it always also hits a sufficient portion of the delay zone to offset the advance. Delays are predominant in the PRC for species such as *M. musculus* (Daan and Pittendrigh, 1976b). Thereby delays become more important in the final response of the system, and an 18-h pulse does not generate phase advances anymore.

The saturation of the phase-shifting action of light needs some further elucidation. This has so far been discussed solely with respect to brief light pulses. Nelson and Takahashi (1991) demonstrated in hamsters that the phase advance produced by a light stimulus of 5 min evokes nearly the same response as a 1-h stimulus. They explained this by presuming that saturation occurs. This may well be true for the instantaneous action of brief pulses. If after a saturating light-pulse light continues to be present for further hours and no complete (type-0) resetting has taken place, then this light will illuminate further phases of the cycle and potentially generate additional delays. This is likely to be the case in our finding that maximal delays are reached at 6-h pulses, which is way beyond the saturating durations in the Nelson and Takahashi (1991) study. There may well be 2 limiting processes in which saturation takes place: 1) a short-term process related to the number of

photons hitting a particular phase of the circadian system and 2) the length of the phase illuminated. While the former (rapid) saturation may or may not be associated with the dark-light transition involved, the latter (slow) saturation must somehow reflect the continuous action of light. The presence of tonic effects is consistent with the dependence of the sustained multiunit electrical activity of SCN neurons on the duration of light exposure in a phase-dependent manner (Meijer et al., 1998).

As pointed out by Johnson (1992), the measurement of PRCs for long light pulses (8-16 h) would describe circadian entrainment properties of animals exposed to complete photoperiods. However, *M. musculus* is a nocturnal animal that does not need to be exposed to the complete photoperiod to entrain to the external 24-h zeitgeber. Instead, the traditional view is that such rodents entrain in response to short light pulses at dawn and dusk, which are essentially the same as a skeleton photoperiod (DeCoursey, 1986; Terman et al., 1991).

What Is the Best Phase Reference for Plotting PRCs?

Aschoff (1965) discussed which phase of the signal should be used as a reference when plotting PRCs: the onset, middle, or end of the light pulse. If the duration of the light pulse has no effect on the shift, then there should be a perfect match of curves of different duration of the signal plotted as a function of the *onset* of the light pulse. That is not the case, as was shown in *Drosophila pseudoobscura* by Pittendrigh (1960): pulse duration does determine the size of the shift. Aschoff found in these data the best fit in the PRCs of different light-pulse duration (1/2000 sec, 4 h and 12 h) when they were plotted as a function of the phase at the mid-pulse time. Aschoff (1965) argued that the mid-pulse was the best marker because if one uses the onset or the end of the pulse, then one implicitly assumes that only 1 transition, from dark to light or vice versa, represents the effective stimulus of the signal. By using the mid-pulse, one takes into account that both light transitions may have an influence on the final response of the system.

Johnson (1991, 1992, 1999) has recommended to plot PRCs as a function of the phase at the onset of the light pulse. He argued that at light onset, the stimulus has not yet interacted with and modified the system. Also, the effective duration of the pulse is unknown for many stimuli (especially chemical/drug stimuli), and therefore the midpoint and the end of the pulse are not precisely known. These are sensible arguments, and many authors have followed Johnson's recommendation. Yet others, including Roenneberg and Rehman (1996), Jewett et al. (1997), and Khalsa et al. (2003), have used the mid-pulse as a reference. We propose that for *light* pulses of different duration, the time of mid-pulse should be the best marker.

Correspondence between PRCs plotted with respect to the phase at midpoint demonstrates that light beyond the onset contributes to entrainment. In an unpublished study by Beersma, the PRCs for different pulse durations measured in 4 different species (humans, Polynesian rats, Syrian hamsters, and flesh flies) superimposed best when plotted as a function of the phase at mid-pulse. The same applies to the mouse PRCs shown in this article (Fig. 2), which were now explicitly collected to test this dependence in a single coherent protocol. The best correspondence was actually found when superimposing the PRCs not at 50% (midpoint) but at 36% of the duration of the light pulse (Fig. 4). This suggests that the onset contributes slightly more to the response of the circadian system than the rest of the pulse. The question that arises is how the various parts of the light pulse contribute to the response.

A Role for Light Adaptation?

We approached this issue with a very simple phase-only approach. We divided each light pulse (3, 4, 6, 9, 12, and 18 h) in portions of 1 h, and then calculated, using the 1-h pulse PRC, the phase of the pacemaker after each hour as if the hour of light exposure had been applied as a single 1-h pulse, preceded and followed by darkness. Implicit in the approach is the assumption that the initial phase shift is indeed completed after 1 h. There are indications that a 2nd pulse presented briefly after a 1st pulse has reduced phase-shifting efficacy (Best et al., 1999). Data from Wong et al. (2005) show that light adaptation of retinal ganglion cells seems to be complete well within 1 h. It is likely that light adaptation plays a role in the reduction of light responsiveness. We therefore assumed that adaptation reduced the efficacy of the light response by a factor x relative to the 1-h pulse. This is similar to the input signal under adaptation as modeled for humans by Kronauer et al. (1999). Hence $x = 1.0$ for the 1st hour of light. For all subsequent hours of light, factor x was optimized as a single factor for each available PRC

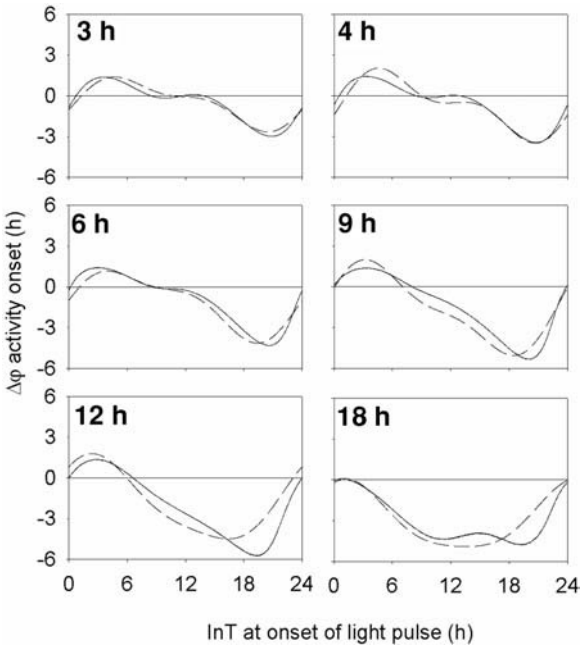

Figure 5. Comparison between actual PRC data (broken lines) and the results of model calculations (continuous lines). The model calculations are performed by accumulating the phase shifts per hour of light exposure as follows: For the 1st hour, the 2-harmonic regression is used as obtained for the 1-h light-pulse PRC. For each subsequent hour, a percentage (22%) of the response as deduced from the same PRC is added. The value of 22% is the one that yields the best correspondence between data and model. InT = internal time.

beyond the 1-h PRC. For example, for the 3-h light-pulse PRC,

$$\phi_2 = \phi_1 + \Delta\phi\,(\phi_1) + [x.\Delta\phi(\phi_1 + \Delta\phi(\phi_1) + 1)] + [x.\Delta\phi(\phi_1 + \Delta\phi(\phi_1) + 1 + x.\Delta\phi(\phi_1 + \Delta\phi(\phi_1) + 1) + 1]$$

$$\Delta\phi = \phi_2 - \phi_1.$$

ϕ_1 = initial phase from 0 to 24 h; $\Delta\phi(\phi)$ = phase shift measured in the 1-h PRC at phase ϕ; and x = reduction factor (0-1) of light effect during later hours relative to light in the 1st hour.

For each PRC with light pulses larger than 1 h, we varied x from 0 to 1 with 0.01 resolution and computed for each x value the total deviance between the PRC thus constructed and the real data measured. The optimal fit (minimal deviance) was obtained with the following reduction factors: 0.15 (3-h pulse), 0.23 (4-h), 0.22 (6-h), 0.23 (9-h), 0.19 (12-h), and 0.30 (18-h). The average reduction factor is thus 0.22, with the encouragingly small standard deviation of 0.045. Also a simultaneous fit to all PRCs yielded a general reduction factor $x = 0.22$. Figure 5 demonstrates that this value indeed yields a reasonable match to all PRCs. Of course, we might have varied x within pulse with consecutive hours. This would entail a near infinite number of combinations to probe and not yield biologically meaningful results. However, we did want to explore whether the last hour of light contributes differently. Keeping for all other hours after the first and before the last in a pulse the reduction factor x at the empirical value of 0.22, we varied the reduction factor for the last hour (y) from –1 to +1. The best fit was obtained when the last hour contributed on average with a factor of $y = 0.20$. Clearly, the value of 0.20 is statistically not distinguishable from 0.22, meaning that in the optimal model, the last hour of the light does effectively the same as the previous hours in a long light pulse. Thus, the data do not suggest that there is a special role for the last hour of the pulse, which might be presumed if the transition from light to darkness itself played a special role in entrainment. In spite of the correspondence of the downward slopes of the PRCs when plotted relative to the offset of the light pulse (Fig. 2C), which might suggest a special role for lights-off, the data are fully compatible with absence of such a role. This is a curious and unexpected result. It does not disprove an effect of lights-off, but it shows that the data do not demand such an effect.

We next assume for a moment that in the simplistic phase-only model, each hour after the first in a light pulse generates a phase shift derived from 0.22 times the measured 1-h phase shift. For each light-pulse duration, it is then possible to calculate the center point of gravity of the light action in this model. For instance, in the 3-h pulse, it is at

$$((1 * 1 + 0.22 * 2 + 0.22 * 3)/(1 + 0.22 + 0.22) - 0.5) / 3 = 0.32.$$

The grand average of the center points thus found for all pulse durations (3-, 4-, 6-, 9-, 12-, and 18-h light pulses) was 0.38 of the duration of the pulse. This fits well with the optimal phase reference point at which PRCs compare best (0.36; see Fig. 3). Apparently, a phase-only model, even though obviously a crude simplification of reality, is sufficient to understand much of the phase-resetting behavior of the pacemaker as long as we account adequately for the effects of light adaptation. The onset of the light pulse has the strongest contribution to the phase shift. The following hours of light contribute less to the phase shift, and no special role is suggested for

the end of the pulse. This analysis conforms closely to the conclusion of Daan and Pittendrigh (1976b) and Daan (1977) that the change in circadian period in LL compared to DD can be derived from the light-pulse PRC if one takes photoreceptor adaptation into account. These authors estimated the adaptation factor to be in the neighborhood of 0.12 to 0.18 (Daan, 1977; Figs. 3,4). That this estimate is smaller than our 0.22 is readily explained by the fact that we use a 1-h PRC as the basis, while they used 15-min PRCs.

Despite other evidence for a more complex structure of evening and morning components of circadian pacemakers (e.g., Pittendrigh and Daan, 1976b; Jagota et al., 2000; Stoleru et al., 2004; Hazlerigg et al., 2005), the data on PRCs presented here are fully consistent with a simple phase-only single oscillator system if adaptation is taken into account.

CONCLUSION

The analysis seems to support the notion that the light throughout the pulse contributes to the steady-state phase shift, but with a slight predominance of the early part of the pulse, possibly due to light adaptation of the circadian system including its photoreceptors. The precise assessment of the effect of the light between the onset and end of the pulse awaits measurements of the responses to comparable skeleton pulses, which we discuss in a subsequent article. For now, it is likely that the light exerts its effect throughout the duration of the pulse. It is also likely that—as proposed by Aschoff in 1963—both parametric and nonparametric effects exert a role in the final response of the circadian system. These standard series of PRCs and τRCs in response to different light-pulse durations contribute to an improved understanding of entrainment.

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