Circadian Phase Resetting in Response to Light-Dark and Dark-Light Transitions

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Abstract Phase shifting of circadian systems by light has been attributed both to parametric effects on angular velocity elicited by a tonic response to the luminance level and to nonparametric instantaneous shifts induced by a phasic response to the dark-light (D > L) and light-dark (L > D) transitions. Claims of nonparametric responses are partly based on “step-PRCs,” that is, phase response curves derived from such transitions. Step-PRCs in nocturnal mammals show mostly delays after lights-on and advances after lights-off, and therefore appear incompatible with phase delays generated by light around dusk and advances by light around dawn. We have pursued this paradox with 2 experimental protocols in mice. We first use the classic step-PRC protocol on wheel running activity, using the center of gravity as a phase marker to minimize the masking effects of light. The experiment was done for 3 different light intensities (1, 10, and 100 lux). D > L transitions evoke mostly delays and L > D transitions show no clear tendency to either delay or advance. Overall there is little or no circadian modulation. A 2nd protocol aimed to avoid the problem of masking by assessing phase before and after the light stimuli, both in DD. Light stimuli consisted of either a slow light intensity increase over 48 h followed by abruptly switching off the light, or an abrupt switch on followed by a slow decrease toward total darkness during 48 h. If the abrupt transitions were responsible for phase shifting, we expected large differences between the 2 stimuli. Both light stimuli yielded similar PRCs characterized by delays only with circadian modulation. The results can be adequately explained by a model in which all PRCs evoked by steps result in fact from tonic responses to the light following a step-up or preceding a step-down. In this model only the response reduction of tonic velocity change after the 1st hour is taken into account. The data obtained in both experiments are thus compatible with tonic velocity responses. Contrary to standard interpretation of step-PRCs, nonparametric responses to the transitions are unlikely since they would predict delays in response to lights-off, advances in response to lights-on, while the opposite was found. Although such responses cannot be fully excluded, parsimony does not require invocation of a role for transitions, since all the data can readily be explained by tonic velocity (parametric) effects, which must exist because of the dependence of $\tau$ on light intensity.

Key words circadian clock, phase resetting, phase response curve, Mus musculus

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The succession of day and night is the most important environmental cycle that synchronizes mammalian circadian rhythms. The relevant key stimuli for entrainment are still incompletely understood. In the early days of biological rhythm research, there were 2 distinct approaches to entrainment. These are related to phasic and tonic responses of the system to the transitions and to the light in between, respectively (Daan, 1977). Jürgen Aschoff (1964) and Rütger Wever (1966) emphasized parametric (tonic) effects of light in their models of entrainment, that is, effects requiring a change in a parameter in the oscillator. Experimentally such effects on the angular velocity of oscillators were primarily derived from the changes in period (τ) with the intensity of constant illumination. Colin Pittendrigh (e.g., 1960, 1981) advocated the non-parametric (phasic) action of light, that is, only an instantaneous resetting of phase without affecting a parameter of the system. He proposed that light-dark transitions rather than the tonic effect of light were the key signals for entrainment. Pittendrigh’s model was based on the experimental assessment of phase response curves describing the responses to brief light perturbations as a function of circadian phase. The implication was that such responses are non-parametric and primarily the consequence of the light-dark and/or dark-light transitions in the pulse. Studies testing the validity of this approach for entrainment to light-dark cycles have been generally positive for short—although not for long—photoperiods (Pittendrigh and Daan, 1976; Comas et al., 2007). In spite of the general acceptance of Pittendrigh’s instantaneous phase shift model, we still do not know which are the key elements in the entrainment of circadian systems respond: phasic responses to the onset (dark-light transition), and/or tonic responses to the light in between (Aschoff, 1965; Kramm, 1971; Beersma et al., 1999).

Step phase response curves (type III in Aschoff’s 1965 classification of PRC protocols) have been used to investigate the potential effects of the transitions separately. In these simple protocols, light is turned on (step-up, D > L) once or is turned off (step-down, L > D) once after a prolonged free run in DD and LL, respectively. The phase shift generated is plotted as a function of the circadian phase at which the switch occurred. In spite of the importance of the questions that might be studied by using this protocol, it has been applied only rarely. Aschoff (1999) restated that step-PRCs have been neglected and suggested that an extensive comparative study on shape and variability of step-PRCs could be helpful for understanding

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**Figure 1.** All previously published data on mammals step phase response curves. Bat and hamster are adapted from Daan and Aschoff (2001, Fig. 22). Phase responses are plotted against internal time at midpulse (InT 18 = activity onset). The responses to dark-light (D > L) and light-dark (L > D) transitions lead to opposite responses in diurnal mammals. Nocturnal mammals (Mesocricetus auratus and Taphozous melanopogon) show mainly delays in response to D > L transition and advances in response to L > D transition. The opposite occurs in the diurnal mammal (Ammospermophilus leucurus). They all show circadian modulation of their response to transitions except for Ammospermophilus leucurus in the L > D transition. (Note: The hamster data in Daan and Aschoff, 2001, were plotted against incorrect circadian time; the abscissa in Figure 22 there should read for hamsters CT 12, 18, 0, 6, 12.)
entrainment. The protocol has been used in beetles (Tenebrio molitor; Lohmann, 1967), bats (Taphozous melanopogon; Subbaraj and Chandrashekaran, 1981), Syrian hamsters (Mesocricetus auratus; Albers, 1986; Aschoff, 1994), antelope ground squirrels (Ammospermophilus leucurus; Kramm, 1974), finches (Fringilla coelebs; Aschoff, 1965), and starlings (Sturnus vulgaris; Subbaraj, 1990).

The mammalian data are summarized in Figure 1, expanding on Daan and Aschoff (2001, Fig. 22). In most of these cases there was a strong dependence of \( \Delta \phi \) on the circadian phase at which the step-up or step-down occurred. In the nocturnal species (M. auratus and T. melanopogon), mostly delays (negative \( \Delta \phi \)) were found following a transition from dark to light (step-up) and mostly advances (positive \( \Delta \phi \)) following a transition from light to dark (step-down). Kramm (1974) obtained in the diurnal squirrel (A. leucurus) mostly advances after a step-up and mostly delays after a step-down. Albers (1986, Fig. 6) combined the phase shifts in hamsters according to the step-up and the step-down PRCs to predict the phase responses to a 15' light pulse. He indeed did obtain curves similar (apart from the absence of phase delays) to brief light pulse PRCs. These findings were considered evidence for an important role of both transitions for entrainment (Albers, 1986).

We recently measured PRCs for light pulses of different durations in mice (Comas et al., 2006). Our data did not support the nonparametric model where only the transitions cause the phase shifts. To explain the differences between PRCs for pulses of different duration we needed to include a tonic effect of the light between the transitions. While the model supported an initial major (either phasic or tonic) contribution of the 1st hour of the light pulse we could not find evidence for any particular role of the end of the light pulse. These conclusions are inconsistent with Pittendrigh's ideas on the role of the light-dark transitions, although a phasic response to lights-on is still an option. To resolve this issue, it would be preferable to have a more direct experimental assessment of the effect of both transitions in step-PRCs.

There is a major hurdle in the interpretation of step-PRCs: the fact that the phases compared before and after the step are assessed in different light conditions. Aschoff (1965) already pointed out the difficulties of calculating the phase shift in this protocol due to the changes in the \( \alpha: \rho \) ratio that result from the changes in light intensity. He therefore proposed to use the midpoint of activity as a reference to calculate the phase shifts. Later authors (including Aschoff, 1994) did not follow this advice and used the onset of activity instead. If light suppresses activity due to masking, as it typically does in nocturnal animals, activity onset during light may well occur at a later circadian phase than during darkness. Thus what looks like the same phase in DD and LL may actually be different phases.

We have returned to the problem of direct assessment of step-up and step-down phase shifts in 2 attempts in Mus musculus. We used 2 different experimental approaches. In the 1st experiment we used the classic step-PRC protocol with different light intensities and using running wheels to record locomotor activity. We further assessed phase by center of gravity (CoG; Kenagy, 1980; Spoelstra et al., 2004) following Aschoff's reasoning (Aschoff, 1965) to reduce the masking problem, and phase shift by state-of-the-art quantitative assessment. In the 2nd experiment we attempted to overcome the problem of masking by assessing phase in continuous darkness (DD) both prior to and after the stimulus. The light stimulus in this case consisted of (1) an abrupt step-up from dark to light followed by a slow ramp-down to DD over 48 h, or (2) a slow ramp-up from DD over 48 h followed by a step-down to darkness. This approach was based on the possibility that the very gradually changing light intensity during the ramps might have negligible overall phasic effects. The difference between the 2 resulting step-PRCs would then be fully attributable to differences between the step-up and step-down, and the masking problem removed. The experiments did not provide convincing evidence for any phasic effect attributable to the transitions. They rather yield the demonstration that all of the responses are compatible with tonic velocity effects.

### MATERIALS AND METHODS

#### Animals and Maintenance

A total of 144 male wild-type C57BL/6JOlaHsd mice (Mus musculus), 1 month old, were obtained from Harlan (Horst, The Netherlands). All mice were housed individually in Lucite cages (25 × 25 × 40 cm) with food (Hope Farms standard rodent pellets, Arie Block, Woerden, The Netherlands) and water ad libitum in a sound attenuated and acclimatized room (temperature 23 ± 1 °C and humidity 60%). The activity was recorded with running wheels (14 cm diameter) connected to an event recording system and stored in 2-min intervals. All cages had equal distance (70 cm) from the light source. After 6 days in DD, the animals were randomly assigned to different experimental conditions to be performed in a randomized order (CoG; Kenagy, 1980; Spoelstra et al., 2004).

#### Experimental Protocols

The protocol has been used in beetles (Tenebrio molitor; Lohmann, 1967), bats (Taphozous melanopogon; Subbaraj and Chandrashekaran, 1981), Syrian hamsters (Mesocricetus auratus; Albers, 1986; Aschoff, 1994), antelope ground squirrels (Ammospermophilus leucurus; Kramm, 1974), finches (Fringilla coelebs; Aschoff, 1965), and starlings (Sturnus vulgaris; Subbaraj, 1990).

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Experiment 1: Step-Up (D > L) and Step-Down (L > D)

In experiment 1 we studied the phase responses to single light transitions in the simple classic way of exposing mice with running wheels to lights switched on or off at different phases of their circadian cycle. Three groups of 48 mice each were kept in complete darkness (DD) for 14 days. On day 15, the 1st group of 48 animals experienced a step-up in light intensity from 0 to 1 lux, the 2nd group a step-up from 0 to 10 lux, and the 3rd from 0 to 100 lux; 14 days later the step-down to 0 lux occurred for all mice, which then stayed in DD for another 14 days.

Experiment 2: Ramp Up–Step Down and Step Up–Ramp Down

A total of 96 mice were kept in continuous dark (DD) for 14 days. After that, half of the mice received a ramp up–step down treatment consisting of a linear increase of light intensity in steps of ~1.05 lux every 2 min until 1500 lux was reached. This took 48 h. After that, lights were turned off (step down from 1500 to 0 lux) and the animals remained in darkness for 14 days. The other 48 mice underwent a step up–ramp down protocol. Here lights (1500 lux) were turned on and after that, with a rate of ~1.04 lux every 2 min, light intensity decreased linearly during 2 days followed by darkness for 14 days. The same experiment was repeated but with a maximum light intensity of 15 lux in both cases (ramp up–step down and step up–ramp down). The rate of change, either increasing or decreasing, was ~0.01 every 2 min during 2 days. By distributing the timing of the 48-h light stimulus over the 24 clock h (1 box with 4 mice starting every 2 h) we spread the signals over the circadian cycles of the mice.

Data Analysis

Phase shifts were calculated by a quantitative and objective procedure as described by Spoelstra et al. (2004). This procedure determines the phase ($\phi_2$) in the cycle at which the light stimulus or step occurred by forward extrapolation from the rhythm before the light stimulus or step and the phase ($\phi_1$) of the same event calculated by backward extrapolation from the rhythm after the light stimulus or step. These phases were derived from $\tau_1$ and $\tau_2$, the periods before and after the pulse, quantified by periodogram analysis over 10 days of activity, excluding the 1st 2 days after the light stimulus or step 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 (CoG minus half-t), that the profile exceeded the overall mean activity, and was set at internal time (InT) 18 (which corresponds to CT 12; see Daan et al., 2002). The phase shift calculated is simply $\Delta \phi = (\phi_2 - \phi_1)$. The phase shifts (in circadian hours) were plotted as a function of the phase at the time of onset of the light transition. Phase response curves (with standard errors) were fitted to the data based on harmonic regression analysis of non-equidistant data, taking 2 harmonics into account, and using the CircWave analysis tool (developed by R.A. Hut; available from http://www.euclock.org/; see also Oster et al., 2006). The number of harmonics that contributed significantly to the explained variance is detailed in Table 1.

Phase Marker: Onset or Center of Gravity?

The phase marker chosen to calculate phase shifts has an effect on the resulting phase response curves (Aschoff, 1965) and their precision. The standard procedure has become to opt for the onset of activity. This is related to the fact that in rodents usually running wheels are used to assess the activity rhythm, and with wheel running the onset of activity is typically the most precise marker in DD (e.g., DeCoursey, 1960): Wheel running onset is...
often subject to less cycle-to-cycle variance than wheel running end or CoG (Daan and Oklejewicz, 2003). Thus for our experiment 2, assessing phase in DD before and after the step, wheel running onset is the best marker. Yet when assessing phase in LL and DD, as in experiment 1, the problem of masking prevails.

Figure 2A and B show 2 examples of the actograms obtained after step-up (0 to 100 lux) and step-down (100 to 0 lux). Lower activity and a smaller $\alpha: \rho$ ratio characterize the activity of the mice during LL in comparison with DD. Figure 2C shows the averaged activity patterns (smoothed by a 10-min running mean) during DD (black line) and LL (gray line). The black and gray dots indicate the onset, CoG, and end of activity during DD and LL, respectively. The activity profiles are lined up using the CoG. In Figure 2A the $D > L$ transition occurs at $\text{InT} \ 5.6$ and the phase shift is -4.22 h for the onset of activity, -5.35 h for CoG, and +2.24 h for end of activity. In 2B the $L > D$ transition occurs at $\text{InT} \ 17.4$ and causes an advance of +2.97 h in the onset of activity, +3.02 h for CoG, and +0.38 h for the end of activity. These different results can be explained by the masking effects that reduce the $\alpha: \rho$ ratio when light is present. To circumvent the different effects of masking we use for experiment 1 both the onset and the CoG and compare the resulting phase response curves in Figure 3.

RESULTS

In 44 of 380 measured phase shifts (~11.5%) a fitted activity onset either before or after the light stimulus was in clear disagreement with visual inspection of the actogram. In these cases the data were excluded from the analysis. Overall, a total of 336 phase shifts were measured in the 2 experiments (Table 1). All PRCs obtained are type 1. In experiment 1, the phase shifts are accompanied by the $\tau$ changes that characterize step response curves: $D > L$ transitions result in lengthening of $\tau$ and $L > D$ transitions result in shortening of $\tau$.

### Table 1. Summary data.

<table>
<thead>
<tr>
<th>Transition (lux)</th>
<th>Number of shifts</th>
<th>PRC amplitude of the fitted curve (fundamental wave + significant harmonics)</th>
<th>Maximum delay of the fitted curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D &gt; L$ (1) CoG</td>
<td>30</td>
<td>(0)</td>
<td></td>
</tr>
<tr>
<td>$L &gt; D$ (1) CoG</td>
<td>31</td>
<td>(0)</td>
<td></td>
</tr>
<tr>
<td>$D &gt; L$ (10) CoG</td>
<td>22</td>
<td>(0)</td>
<td></td>
</tr>
<tr>
<td>$L &gt; D$ (10) CoG</td>
<td>24</td>
<td>(0)</td>
<td></td>
</tr>
<tr>
<td>$D &gt; L$ (100) CoG</td>
<td>26</td>
<td>(0)</td>
<td></td>
</tr>
<tr>
<td>$D &gt; L$ (100) onset</td>
<td>32</td>
<td>1.31 (1)</td>
<td>2.18</td>
</tr>
<tr>
<td>$L &gt; D$ (1) onset</td>
<td>35</td>
<td>0.87 (2)</td>
<td>0.04</td>
</tr>
<tr>
<td>$L &gt; D$ (10) onset</td>
<td>24</td>
<td>0.91 (1)</td>
<td>-1.06</td>
</tr>
<tr>
<td>$L &gt; D$ (100) onset</td>
<td>27</td>
<td>(0)</td>
<td></td>
</tr>
<tr>
<td>$L &gt; D$ (100) onset</td>
<td>32</td>
<td>1.22 (1)</td>
<td>1.68</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Light stimulus (lux)</th>
<th>Number of shifts</th>
<th>PRC amplitude of the fitted curve (significant harmonics)</th>
<th>Minimum delay of the fitted curve</th>
<th>Maximum delay of the fitted curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\uparrow$ (15) onset</td>
<td>41</td>
<td>0.89 (1)</td>
<td>-2.74</td>
<td>-4.52</td>
</tr>
<tr>
<td>$\uparrow$ (15) onset</td>
<td>35</td>
<td>0.74 (1)</td>
<td>-2.42</td>
<td>-3.91</td>
</tr>
<tr>
<td>$\uparrow$ (1500) onset</td>
<td>40</td>
<td>1.93 (2)</td>
<td>-3.96</td>
<td>-7.82</td>
</tr>
<tr>
<td>$\uparrow$ (1500) onset</td>
<td>37</td>
<td>1.30 (1)</td>
<td>-5.26</td>
<td>-7.86</td>
</tr>
</tbody>
</table>

NOTE: For each dark-light ($D > L$) or light-dark ($L > D$) transition (experiment 1) or light stimulus (experiment 2) the number of shifts that were measured and used for analysis is specified. For each phase response curve, based on harmonic regression fits, the maximum delay and advance (in hours) and the corresponding amplitude defined as half the distance between maximum advance and delay are indicated. CoG = center of gravity.
Experiment 1: Step-Up (D > L) and Step-Down (L > D)

Figure 3 (A-L) shows the phase responses obtained for the D > L and L > D transitions for 1, 10, and 100 lux. The phase shifts obtained for each transition and light intensity are plotted twice using either the onset of activity or the CoG as phase references. D > L transitions elicit delays and L > D transitions elicit both advances and delays. For step-ups, harmonic regression yielded evidence for circadian modulation in phase shifts obtained with the D > L 1 lux, D > L 10 lux, but only when the onset of activity was used (Fig. 3A, E). This demonstrates that the evidence for circadian variation in the phase shifts is dependent on the phase reference used. For step-downs, harmonic regression yielded evidence for circadian modulation only for the L > D 100 lux step, independently of the phase reference used (Fig. 3K, L). Maximum delays in the phase-dependent step-up PRCs and maximum advances in the step-down PRCs both occur approximately in the middle of the subjective day (InT 6-18), confirming the published data shown in Figure 1. This is in sharp contrast to light pulse PRCs that show minimal shifts in the mid-subjective day (“dead zone”).

Experiment 2: Step Up–Ramp Down and Ramp Up–Step Down

Figure 4 summarizes the PRCs obtained in experiment 2. If transitions are a key stimulus, we expect to find large differences between the 2 stimuli since 1 involved a step-down, the other a step-up transition. As can be noted in Figure 4, only delays were obtained with both types of light stimuli. There are no major differences between PRCs obtained with the 2 types. In both figures, larger delays are observed when the transitions occur during the subjective day (InT 6-18) than when they occur in the subjective night (InT 18-6). When the maximum light intensity used is 1500 lux, the average delay was -6.33 h for step up–ramp down and -6.56 h for ramp up–step down (Fig. 4C, D).

Reducing the maximum light intensity to 15 lux (Fig. 4A and B) yielded similar results as with 1500 lux (Fig. 4C and D). Only delay phase shifts were obtained and no advances. The amplitude is approximately half the amplitude obtained with 1500 lux.
The delays averaged –3.63 h for step up–ramp down and –3.17 h for ramp up–step down.

**DISCUSSION**

Step-up and step-down phase response curves have been used previously to study the contribution of each light-dark transition to entrainment. Albers (1986) even attempted to reconstruct the PRC for brief light pulses in hamsters from their responses to lights-off and lights-on measured in step-PRCs separately. Such step-PRCs would be evidence for phasic (nonparametric) entrainment responses to the transitions if the light did not have other effects such as tonic (parametric) responses and masking.

However, all step-PRC data published are in disagreement with the results obtained by entrainment and light pulse PRCs. There is first a curious fact that has so far escaped the discussion of these data. The nocturnal species behave opposite from what one would expect on the basis of entrainment principles: step-PRCs mostly show delays following lights-on and advances following lights-off (Figs. 1, 3). If the transitions are the key elements in natural entrainment one would expect that lights on (which corresponds to dawn) would generate a phase advance, while lights-off (corresponding to dusk) would generate a phase delay. The opposite is true.

Second, in diurnal animals (restricted to data sets on a ground squirrel in Kramm, 1974, and a finch in Aschoff, 1965) phase shifts to steps yield results (Fig. 1) opposite from the nocturnal species. This is despite that (1) nocturnal and diurnal mammals have circadian pacemakers in synchrony with each other both in terms of electrophysiology (Sato and Kawamura, 1984) and gene expression (e.g., Mrosovsky et al., 2001; Dardente et al., 2002), and (2) their PRCs are in phase with each other and indeed virtually identical (Daan, 2000, Fig. 9).

Third, there is circadian modulation with reduced responsiveness during the subjective night (Figs. 1, 3). This is the opposite of the maximal responsiveness to light pulses observed in the subjective night.

Thus, the published data themselves yield strong evidence that what they measure is not the instantaneous phase shift of the pacemaker in response to the L > D or D > L transitions as they aim to measure. We are apparently looking at other effects of light, and it may be difficult to disentangle their relative contributions. In the 1st place, changes in \( \tau \) due to LL (Fig. 2A and B) might have led to miscalculation of the phase.
shifts. The tonic response to light before or after the steps may have resulted in overall phase shifts not attributable to the steps themselves. This tonic light influence accumulates over long episodes of the light (Comas et al., 2006) and thus may lead to an apparent phase shift of the system. Second, masking effects (Fig. 2C) of light may cause the onset of activity to occur at a later circadian phase than during darkness. Thereby, when focusing on activity onset, researchers may have observed delay shifts after a D > L transition and advance shifts after the light is turned off (L > D), without the pacemaker being affected by the transitions themselves.

In experiment 1 we minimized the problems of miscalculation and of masking by measuring step-PRCs in Mus musculus using 3 different light intensities (1, 10, and 100 lux), and by using the CoG, in addition to the activity onset, as phase marker. Since we cannot be sure that masking is fully eliminated, we showed both phase markers for comparison in Figure 3. The results are not dramatically different, and possibly masking is a lesser problem than we anticipated. What remains is the possibility that slow changes in the tonic velocity effect of light cause the apparent phase shifts due to the D > L transitions. The PRCs (Fig. 3) show that D > L transitions in mice mostly lead to delays and L > D transitions to no shifts. The phase shifts do not appear to depend on circadian phase of the activity rhythm, except very slightly at the high light intensity of 100 lux.

The average circadian period for these mice was 23.73 (±0.20) h in DD and 25.4 (±0.17) in LL (100 lux). Using the tonic velocity response curve approach, in which a light pulse PRC is multiplied with a reduction factor and then used to compute progress of circadian phase per hour (Comas et al., 2006), we find that a reduction factor of 0.16 is needed to obtain this change in free-running period from the 1-h phase response curve. This is smaller than the factor of 0.22 predicted from comparing effects of light of up to 18-h duration. This difference suggests that the response reduction during light exposure may actually be a slower process than assumed earlier (Comas et al., 2006). If that is the case, then the 1st 1 or 2 cycles in LL will be slowed down more than later cycles. Since the phase shift is measured by extrapolating back from those later cycles, the increased initial parametric slowing of the system will lead to substantial apparent delay phase shifts, nearly regardless of the circadian phase at which the light is turned on. Thus the data from our experiment 1 are sufficiently explained by tonic responses to the light during LL leading to apparent delay shifts, and do not prove the existence of phasic responses to the light-dark transitions themselves.

Even if masking is minimized in our analysis, it is unavoidably present in step-PRC data. Therefore, we measured phase response curves using ramp up–step down and step up–ramp down light profiles in experiment 2. Contrary to our expectations, all of the ramp/steps yielded only phase delays, with large average delays and circadian amplitude for 1500 lux and small average delays and amplitude for 15 lux. The circadian modulation for both stimuli is in phase, not in antiphase as would be expected if the step up and step-down transitions would cause them. It indeed hardly matters for the results whether the step was up or down. It is best comparable to the response to a step-up transition in experiment 1 in both cases. It
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is, therefore, most likely that these delays are not primarily due to the transitions but to the light during the slow ramp-up or ramp-down. There is again no evidence for a phasic (nonparametric) transition effect.

We pursue in some more depth the idea that the light acted tonically before or after the transitions to yield the effects observed (Fig. 5). We again make use of the 1-h light PRC determined by Comas et al. (2006) and the same phase-only model with response reduction described there. We assume the effect of each hour of light is reduced by a constant factor and calculate which reduction factor leads to the best fit of the overall phase shifts generated by the model to the 2-harmonic curve fitted to the data. Implicit in the approach is the assumption that the very slow changes in intensity are likely to lead to similar responses for each additional hour despite the gradual changes in intensity. This must be a very coarse simplification of reality, but it at least allows us to generate a prediction. For the step up–ramp down we assume a full effect of the 1st hour and constant response reduction from then onward. The best fit is obtained with a factor of 0.20 from the 2nd till the 48th hour of the light stimulus. In the ramp up–step down we do not assume a special effect for the 1st hour because the initial light intensity is extremely low. The best fit is obtained with a reduction factor of 0.21 for the full 48 h. These response reduction factors around 0.2 are very close to the average reduction factor of 0.22 found for predicted PRCs to light pulses of durations up to 18 h in our previous study (Comas et al., 2006).

CONCLUSION

The response to light steps-up and -down has been attributed previously to an instantaneous phase shift at the moment of the transition. Our analysis (confirming published data) shows that steps-up only induce delays, and steps-down primarily induce advances. If this were due to the phasic response at the transition, lights-on in entrainment should induce delays, and lights-off in entrainment should induce advances. We know this is not the case. Also, sensitivity should be greatest in the subjective night, not in the subjective day where it peaks around internal noon, that is, in the middle of the “dead zone” of minimal sensitivity observed in pulse PRCs. For these strong reasons, the phasic explanation is inadequate to explain our results here, although we cannot fully exclude any participation of phasic effects under all conditions. In contrast, all of our results are fully compatible with, and can be explained by, tonic velocity effects. Clearly parametric effects are both necessary and sufficient for entrainment. This resolves the paradox of phase delays seemingly generated by lights-on and phase advances seemingly generated by lights-off. The step transitions may well play no specific role (see also Nelson and Takahashi, 1991, 1999). In earlier work (Beersma et al., 1999) we have distinguished between phase and period responses, referring to instantaneous phase shifts and aftereffects in response to light, respectively, without implying a response to the transitions.

Functionally, the absence of phasic responses to sudden light-dark transitions would free circadian clocks from being shifted back and forth each time the animals move into and out of dark burrows in daytime. Tonic effects modulated by response reduction in ongoing illumination and response reduction in darkness provide a more stable solution to entrainment. Intensity-dependent tonic effects also allow the perception of twilight gradients in light intensity known...
to be of significant importance in mammalian entrainment (Boulos et al. 2002).

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