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Published in:
Journal of Biological Rhythms

DOI:
10.1177/0748730408330196

Publication date:
2009

The Progression of Circadian Phase during Light Exposure in Animals and Humans

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Abstract Studies in humans and mice revealed that circadian phase shifting effects of light are larger at the beginning of a light exposure interval than during subsequent exposure. Little is known about the dynamics of this response reduction phenomenon. Here the authors propose a method to obtain information on the progression of phase during light exposure. Phase response curves to intervals of light exposure over a wide range in duration are available for flesh flies, mice, and humans. By comparing the phase shifts induced by pulses of various durations but starting at the same circadian phase, the progression of phase during a long interval (hours) of light exposure is reconstructed for each of these 3 species. For flies, the phase progression curves show that light pulses—if long enough—eventually make the pacemaker stabilize around InT18 (near subjective dusk), as is typical for strong resetting. The progression of phase toward the final value never shows advances larger than 7 h, while delays can be as large as 18 h. By applying the phase progression curve method presented in this study, differences between advances and delays in type-0 phase response curves can be distinguished clearly. In flesh flies (*Sarcophaga*) this bifurcation between delays and advance occurs when light exposure starts at InT0 (subjective midnight). The present study confirms earlier findings in mice showing that the beginning of the light pulse generates stronger phase shifts than subsequent hours of light. Response reduction is complete within 1 h of exposure. It is argued that the variation is not so much due to light adaptation processes, but rather to response saturation. In contrast to light adaptation, response saturation is fundamental to proper functioning of the circadian pacemaker during natural entrainment. For understanding entrainment of the pacemaker to natural light, phase progression curves in which naturalistic light profiles are applied could be an important tool.

Key words adaptation, saturation, fly, mouse, human

THE PROGRESSION OF PHASE DURING LIGHT EXPOSURE

Under normal conditions of exposure to light and darkness, the circadian pacemaker is exposed to steeply fluctuating light intensities. For a human being, clouds, shades, retreating in a building, closing one's eyes, and electric lighting all modify light intensity as provided by the sun in major ways (Beersma et al., 1999). The circadian pacemaker uses the whole fluctuating light

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JOURNAL OF BIOLOGICAL RHYTHMS, Vol. 24 No. 2, April 2009 153-160
DOI: 10.1177/0748730408330196

signal for setting its phase. Pacemaker phase will therefore not progress linearly as a function of time of day, but fluctuations must occur in response to the fluctuations in light intensity. For understanding entrainment, and especially how circadian systems cope with the noisy light-dark signal, it is important to understand this progression of phase. In this study we use phase response curves (PRCs) to constant light intensity exposure during intervals of various durations (between 1 and 20 h) to investigate the dynamics of entrainment of the pacemaker.

Rimmer et al. (2000) and Comas et al. (2007) have demonstrated that the phase shifting effects of light are stronger for the beginning of the light pulse compared with the remainder of the stimulus. For mice, Comas et al. estimated that the effects of the 1st hour of light exposure are about 4.5× stronger than the average value for subsequent hours. In this study we describe a method to analyze this type of response reduction as a function of time during light exposure in more detail. In accordance with the observations of Comas et al. (2007) we assume that the transition from light to darkness by itself does not induce a phase shift of the circadian pacemaker, and that the shift of phase therefore is only due to the presence of light compared with darkness.

The problem of understanding the progression of phase of a circadian pacemaker during light exposure is the fact that it is not possible to continuously monitor phase from the overt rhythm such as is apparent in activity records. We usually quantify shifts in phase by using a phase marker (the onset of activity or the onset of melatonin production, for instance) and calculate the difference in timing between actual occurrence of the phase marker with its expected timing. Based on this approach it is possible to indirectly derive the progression of phase during a long light pulse as follows.

First the animal under consideration is kept in constant darkness (DD) until it reaches the phase φ in its activity-rest cycle we are interested in. At that time, only the 1st hour of the intended light stimulation is applied. The response to this 1st part of the light stimulation is quantified in subsequent DD, by comparing the shift in timing of the phase marker relative to a control study in DD. Subsequently, after a few weeks to allow for aftereffects of the pulse to fade away, the identical light is turned on at the same phase φ in the cycle. Now the light is applied for 2 h instead of 1, and again the phase shift is quantified. This procedure is repeated for the first 3 h of the light profile, the first 4 h, and so on. After having performed all these experiments, the progression of phase during the long light exposure interval can be reconstructed from the responses to the applied shorter intervals of the pulse. The curve describing the progression of phase over time we call the phase progression curve (PPC). In principle it is possible to use naturalistic light profiles (including fluctuations) for such investigations, but no data on phase progression during naturalistic light exposure are currently available. What is available, however, are PRCs to rectangular light pulses of different duration. Plotting those available PRCs as a function of the phase at the onset of the pulse allows us to reconstruct PPCs by reading the phase shifts sequentially from the PRCs with increasing pulse duration.

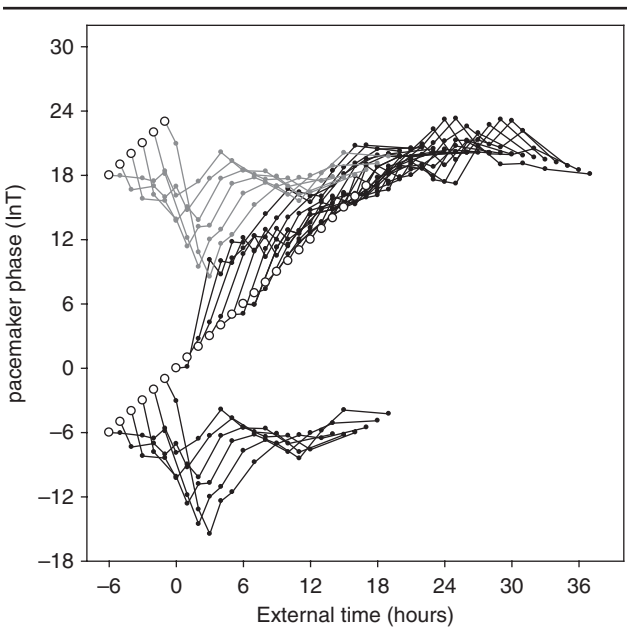

Figure 1. Phase progression curves in *Sarcophaga argyrostoma*, calculated from PRCs published by Saunders (1978). Each open circle represents the start of a light pulse. The actual time at that start (plotted along the abscissa) is taken to be equal to the internal time of the animal (plotted along the ordinate). The open circle is connected to the phase 1 h later. The time along the abscissa increases by 1 h, and the new value of the phase is derived from the PRC to 1-h light pulses. If no response occurred, the phase would have increased by 1 h due to the passage of time. Advances lead to relatively higher values, delays to relatively lower values. The subsequent point in each graph represents the phase after a light pulse of 3 h, derived from the 3-h PRC. Subsequent points represent the phases after 4, 5, 6, 8, 10, 12, 14, 16, and 20 h of light exposure, respectively. The curves in gray are double plots of the 6 lowest curves, to show that all curves end around the same final phase (~InT18, i.e., subjective dusk).

Results of this approach are plotted in Figure 1 for flesh flies (from Saunders, 1978), in Figure 2 for mice (from Comas et al., 2006), and in Figure 3 for humans (several data sources listed in legend of Figure 4). In

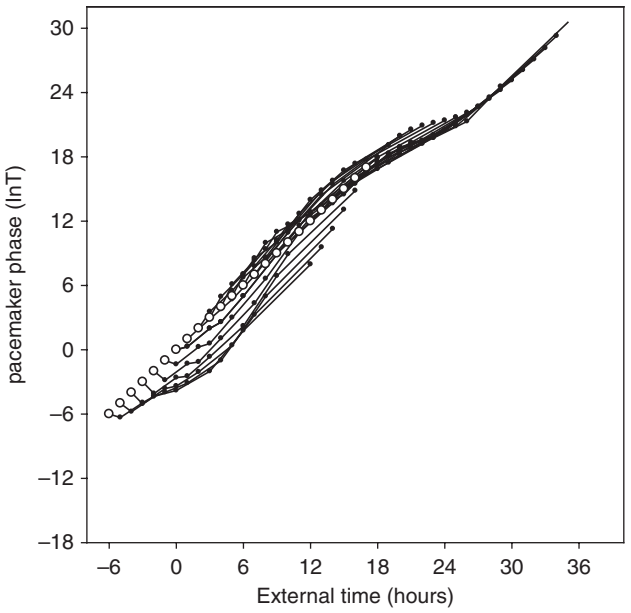

Figure 2. Phase progression curves in *Mus musculus*, calculated from PRCs published by Comas et al. (2006). Light pulses applied are of 1-, 3-, 4-, 6-, 9-, 12-, and 18-h duration.

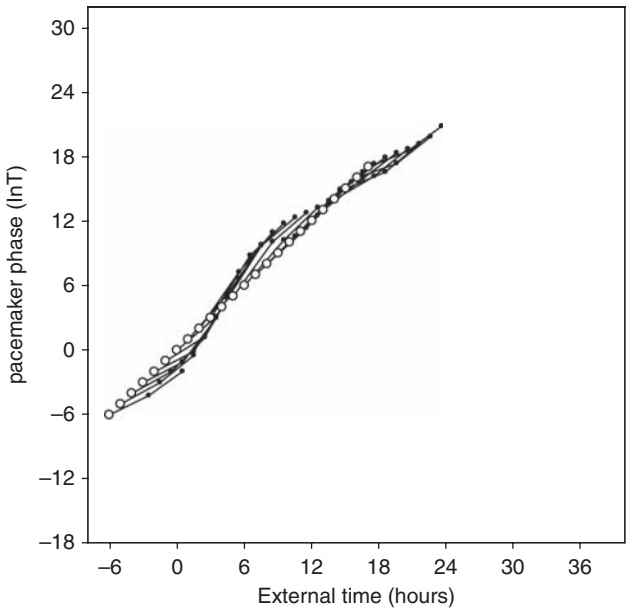

Figure 3. Phase progression curves in humans, calculated from the "short"-pulse PRC as described in Figure 4 and the 6.5-h pulse-PRC by Khalsa et al. (2003). To translate the timing of the light pulses relative to the minimum in core body temperature into InT we have assumed that the minimum of core body temperature occurs at InT1, that is, 1 h after midsleep. Midsleep is taken as ExT0. InT = internal time; ExT = external time.

each of these graphs, the abscissa presents the progression of time in circadian hours, that is, 24 circadian hours correspond to 1 complete cycle of behavior of the animal in DD. The ordinate represents the progression of phase of the behavioral cycle, also in circadian hours. Before the application of the light pulse, internal phase = 0 (InT0) is defined to occur at external time = 0 (ExT0). (InT refers to internal time, which relates to circadian time, but with subjective midnight as the origin of the scale, instead of subjective lights-on. ExT refers to external time, which relates to zeitgeber time, but with midnight as the origin of the scale instead of the onset of the photoperiod. Under natural light-dark conditions ExT equals clock time; see Daan et al., 2002.) Humans have modified the light-dark cycle they perceive to such an extent that midnight is no longer the midpoint of the perceived dark interval. In a sample of 8200 Dutch subjects who completed the Dutch version of the Munich Chronotype Questionnaire (Roenneberg et al., 2003) midsleep (weighed average of work days and free days) occurred at 0407 h. Therefore, we take 0400 h as ExT0 in humans in this study.

Without light application the progression of phase would be equal to the progression of time, and data points would line up along the line $y = x$. If light exposure at a certain time induces an advance on top of the advance due to the passage of time, this is shown as relatively higher values in the figure, and delays show up as values below the diagonal. Figures 1 to 3 each contain a complete series of 24 PPCs in which 24 light pulses each are taken to start at phase angles that successively differ by 1 circadian hour.

For the eclosion rhythm of *Sarcophaga argyrostoma* (Fig. 1) it can be seen that after going through a range of phases, the system always stabilizes at about InT18 (not necessarily exactly at this phase), which corresponds to the beginning of the subjective night. Around this time the light induces delays. If the delays are of a magnitude of 1 h per hour of light exposure, the delays compensate for the advances resulting from the passage of time, and therefore the phase settles to a constant value. This final phase is independent of the phase at which the white light pulse (with an intensity of 240 $\mu W/cm^2$) was started. The result of final phase being independent of initial phase in flesh flies was known for a long time (Saunders, 1978). It is called strong resetting (Winfree, 1970), or type-0 resetting, and it also applies to other insect species, like *Drosophila* (Pittendrigh and Minis, 1964). Some of the PPCs for the flies show that the changes in phase during a light pulse are not always in the same direction. Pulses starting between InT18 and InT0 first show delays and then advances before

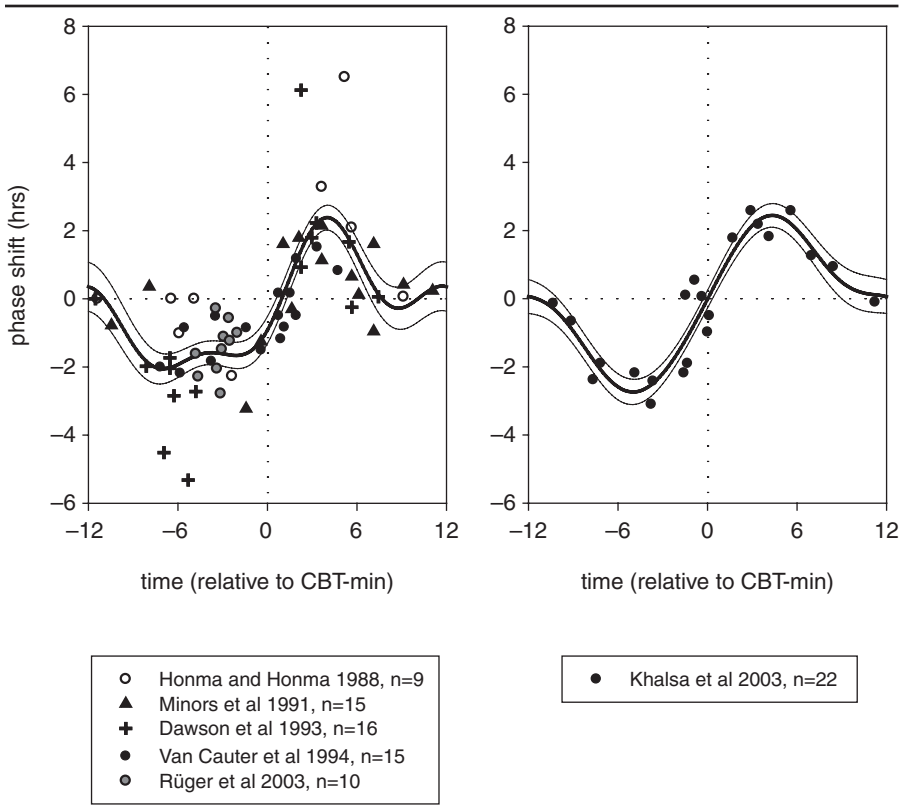

Figure 4. Human phase response curves to light pulses of relatively short (3-4 h) and slightly longer (6.5 h) duration. (a) Studies of 3- to 4-h light pulses. This concerns 9 data points by Honma and Honma (1988), 15 by Minors et al. (1991), 15 by Dawson et al. (1993), 16 by van Cauter et al. (1994), and 10 by Rüger et al. (2003). (b) Results of 6.7-h light pulses. This study concerns 22 data points by Khalsa et al. (2003). All data are plotted relative to the timing of the minimum of core body temperature (CBT). Since the data by Honma and Honma (1988) were originally presented relative to activity onset, those time points needed to be transformed to plot them relative to the minimum of CBT. We assumed that the minimum of CBT occurred 2.7 h before activity onset (see Beersma and Dann, 1993). The phase of light exposure in the data by Rüger et al. (2003) was based on the time at which 25% of maximum melatonin concentration in saliva was reached. In accordance with Khalsa et al. (2003) we assumed that this measure of dim light melatonin onset occurred 7.0 h before the minimum in CBT. The data by Khalsa et al. (2003) are corrected for the estimated drift of the circadian system due to free running in dim light. The intrinsic period of the human circadian pacemaker in dim light has been determined to be, on average, 24.18 h (Czeisler et al., 1999). Since the shifts were measured 3 days after baseline, we added 3× 0.18 h to the phase shifts obtained by Khalsa et al. (2003) to render them comparable with the other data. The 2 resulting data sets of Figure 4 are fitted by (nonequidistant) Fourier analysis in which the 24-, 12-, and 8-h components are taken into account.

stabilizing near InT18. For pulses starting close to InT0, the delays are much bigger than needed to synchronize to dusk. Overshoots can be as large as ~10 h. The final stable phase position (around InT18) is only reached after subsequent advances, which not so much represent responses to the light but are largely just due to the passage of time. The light pulse beginning at InT23, 1 h before subjective midnight, induces the largest delays, of up to 18 h. In contrast there are no PPCs showing advances beyond 7 h.

For *Mus musculus* the PPCs (Fig. 2) are qualitatively different. Long white light pulses of 100 lux do not lead to stable final phase angles. Instead, phase continues to change even under pulses of 18-h duration. This is weak resetting, or type-1 resetting according to Winfree (1970). While PPCs sometimes depart from the $y = x$ line with a steep angle at the beginning of the light pulse, after a while they begin to run in parallel with the line $y = x$. This means that at later times during the light pulse, the phase of the pacemaker mainly progresses proportionally to the progression of time, and the effect on phase of continued light exposure, per se, is small.

Data in humans are not as abundant as in mice or flesh flies. Results of various studies need to be combined to estimate PRCs to relatively short light pulses (see Beersma and Daan 1993, data set extended in Fig. 4). For longer light pulses (6.5 h) data by Khalsa et al. (2003) were used. Under the assumption that the differences in light intensity (ranging from 2500 to 10,000 lux) have little influence on the phase shifts (Zeitzer et al., 2000), it is possible to construct short PPCs for humans (Fig. 3), up to 6.5 h of light stimulation. The results are very different from those of the flesh flies and they are qualitatively similar to the data of the mice. They are type-1 PPCs.

THE IMPACT OF PRIOR LIGHT EXPOSURE

PPCs as derived from comparing the phase shifts elicited by light stimuli of different duration are potentially very useful to assess the effect of prior light exposure. What we need to know is how phase progression in the 2nd hour of light exposure relates

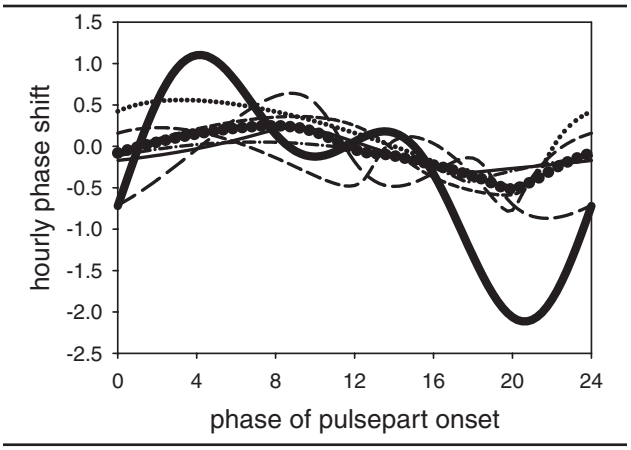

Figure 5. Incremental PRCs for subsequent hours during the light pulse in *Mus musculus* (data by Comas et al., 2006). Phase shifts are plotted as a function of the phase at which the respective hour of the light pulse was applied. The heavy black line represents the smoothed PRC to 1-h light pulses. The thin lines represent incremental PRCs for subsequent intervals of the light pulse. The average incremental PRC for the 2nd and 3rd hour of the pulse is represented by the thin continuous line. The effect of the 4th hour is indicated by large dashes. Slightly smaller dashes indicate the effects of hours 5 and 6; short dashes indicate the average effects of the hours 7 through 9; small dots indicate hours 10 through 12; and the dash-dot-dash line indicates the average effects of hours 13 through 18 of the pulse. The hourly average effect, excluding the 1st hour of the pulse, is indicated by the heavy dots.

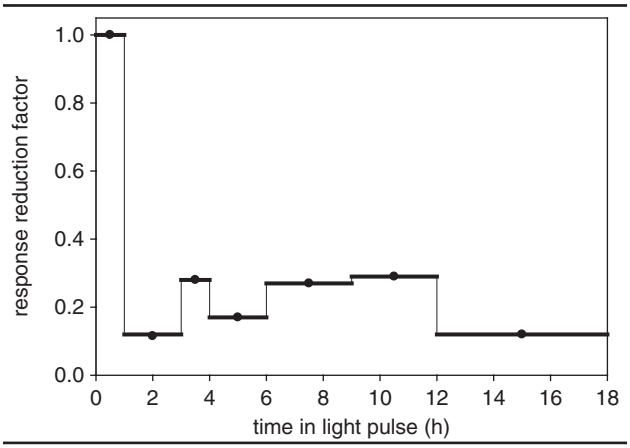

Figure 6. Response reduction as a function of time during light exposure. Values resulted from a linear regression analysis in which the phase shifts calculated for, for instance, the 2nd hour of the pulse are plotted against the phase shifts to the 1st hour, obtained at the same circadian phase.

to phase progression in the 1st hour of light exposure if both start at the same phase.

From the curves in Figures 1 to 3 it is clear that, especially for mice and humans, the phase changes induced by light are larger at the beginning of the light pulse. This notion can be quantified by calculating the incremental effect of each additional hour of light exposure. For that purpose we read the final phase of a light pulse of a certain duration, for example, a 3-h light pulse, from each PPC. This final phase is considered to be the initial phase at which the last hour of a 4-h pulse would start. The result of the 4-h pulse is also read from the PPC. The phase shift induced by the 4th hour is computed as the difference between the final phases after 3 h and 4 h, respectively, and corrected for the passage of 1 h of time.

Figure 5 shows the incremental effects of light on phase per hour of light application as a function of time in the light pulse and as a function of the phase at which the incremental time interval started for mice. The figure confirms and quantifies the impression from the PPCs that phase shifting effects are larger in the beginning of the pulse than at later times, as also shown in Comas et al. (2006). By regressing the incremental PRCs for subsequent hours on the PRC of the 1st hour of light exposure, it is possible to estimate the magnitude of response reduction as a function of time in the light pulse. This is shown in Figure 6. The reduction in responsiveness to light after the 1st hour is conspicuous in mice. The human data are insufficient in number to allow similar computation. Yet, the PPCs suggest that response reduction with increasing pulse duration is also common in humans.

DISCUSSION

The transformation of PRCs for different light pulse durations into PPCs has allowed us to quantify an important aspect of the dynamics of the mammalian circadian pacemaker: the amount by which prior exposure to light reduces the response to subsequent light exposure. This is particularly evident in weak resetting. In strong resetting, Figure 1 demonstrates that the 2nd hour of the pulse frequently yields the largest phase shifts. Simple clocks (these are clocks completely characterized by phase) cannot show strong resetting (Winfree, 1970). Strong resetting can only occur in complex clocks. The complexity may stem from amplitude responses of the clock oscillation, but the complexity can also result from the interaction of many simple clocks that collectively compose the pacemaker (Winfree, 1980). The strong resetting in *Sarcophaga* could thus be due to the fact that the 1st hour of the pulse substantially reduces the amplitude of the oscillation of the pacemaker. Reduced amplitude increases sensitivity to a phase shifting stimulus. As a consequence the influence on phase of subsequent

light increases and phase shifts are larger. Alternatively, the 1st hour of the light pulse could redistribute the phase angles of simple-clock cellular oscillators in the fly's pacemaker, after which the remainder of the pulse can be more effective to reset the composite clock. Irrespective the precise mechanism, the PPCs show which final phases are reached through advances and which through delays. Commonly type-0 PRCs are plotted such that phase shifts range from –12 h to +12 h, and a dotted vertical line is frequently used to suggest at which phase delays change into advances (see, for instance, Figure 3 in Saunders, 1978). From Figure 1 it can be seen that for the eclosion rhythm of *S. argyrostoma* delays can be as large as 17 or 18 h and advances never exceed 6 or 7 h. PPCs determine at which phase the bifurcation between advances and delays occurs. This is when the light pulse starts at InT0. As thoroughly investigated and elegantly mathematically explained by Winfree (1973) for the eclosion rhythm of *Drosophila melanogaster*, a light pulse applied at this time and with exactly the right strength brings the system to the singularity point. After such pulse there is no apparent circadian rhythmicity left. The amplitude of the rhythm is reset to zero. Stronger pulses yield strong resetting; weaker pulses yield weak resetting.

In weak resetting, response reduction has important functional implications, especially in combination with slow response restoration in prolonged intervals of darkness. If an animal retreats into its burrow, for instance, it will not see light and therefore misses the response to the light it otherwise would have seen (Hut et al., 1999). As has been demonstrated experimentally by Rimmer et al. (2000) in humans and Comas et al. (2007) in mice, during darkness the response magnitude is restored almost exactly to the extent that the extra response to subsequent light compensates for what was missed during the dark interval.

An obvious mechanistic interpretation of the phenomena of response reduction and response restoration is that they are attributable to light adaptation and dark adaptation processes. This is unlikely to be completely correct on functional grounds. Light adaptation is a response of the visual system to achieve that the cells are working in their appropriate dynamic range. As a result contrasts can be perceived at a wide range of ambient light intensities (~7 log-units). Adaptation serves essentially to compensate for differences in average light intensity. Upon adaptation, more light is required to yield a similar response, so the response to a specific light intensity is reduced. This causes the intensity-response function to shift to higher light intensities. This is crucial for vision. For pacemaker entrainment, changes in absolute light intensity are needed to be detected. For synchronization with dawn and dusk, the pacemaker somehow needs to detect when light intensity exceeds a certain threshold value, irrespective of the prior history of light exposure.

The input pathway to the SCN indeed seems to be constructed to maximally avoid light adaptation. First, the retinal ganglion cells, assumed to be responsible for a large fraction of the response of SCN neurons, contain melanopsin as the functional photopigment (Berson et al., 2002; Hattar et al., 2002). Melanopsin is considered to be a bistable photopigment (Melyan et al., 2005; Mure et al., 2007). The color composition of the incident light largely determines the ratio between the concentrations of molecules in the 2 states, not light intensity (Walker et al., 2008). Second, the range of light intensities in which the ganglion cells operate begins at lower intensities than the pupil response (Dkhissi-Benyahya et al., 2000; Hut et al., 2008). So the pupil does not modulate the relevant input signal to the circadian pacemaker. Third, anatomically, the melanopsin cells consist of a wide net of thin branches covering substantial parts of the retina (Hattar et al., 2002), thought to be necessary for spatial integration of environmental light intensity. This anatomical structure is very different from the compact structures of rods and cones. In rods and cones the phototransduction at 1 location of the membrane must lead to microelectrical changes that influence subsequent photon capture at nearby locations, simply because membrane currents must extend over a certain volume of the photoreceptor outer segment, which is densely packed with membrane. The loose structure of the ganglion cells, in contrast, may achieve the opposite: almost independent phototransduction effects of different photons. Fourth, melanopsin immunostaining revealed numerous "bead-like" dendritic swellings (Hattar et al., 2002) filled with melanopsin that appear to be unbound to the cellular membrane. These structures may increase the capacity of the melanopsin cells to rapidly replenish their functional membrane-bound melanopsin pool when necessary, hence avoiding reduction in sensitivity. Likely, these mechanisms collectively lead to limited adaptation of the ganglion cells to prior light exposure. Wong et al. (2005) have shown that the intrinsically light sensitive retinal ganglion cells do show adaptation. Under very high light levels they observed adaptation over about 2 log-units. Even if the range of adaptation would be similar for the much lower light intensities to which the circadian pacemaker can respond, such a range of adaptation is small

compared with the adaptation capacity of the visual system. The narrow range of adaptation may help explain the fast transient adaptation of SCN neurons to new levels of exposure followed by sustained and persistent responses almost coding for absolute light intensity (Meijer et al., 1986; Drouyer et al., 2007).

In summary, it seems that light adaptation such as presents in rods and cones is reduced to a minimum in the ganglion cells. Yet, reduced sensitivity of melatonin suppression to nocturnal light has been observed after exposure to bright light on the day(s) before (Hébert et al., 2002; Smith et al., 2004; Jasser et al., 2006). Although those results may indeed demonstrate adaptation to sufficiently bright light, it is also possible that the light applied prior to the test pulses modified the temporal relationships between pacemaker cells, leading to different saturation effects in response to the nocturnal test pulses.

If, at the relevant intensity levels, little light adaptation is present in the input pathway to the circadian pacemaker, the observed reduction in response to sustained light exposure, such as depicted in Figures 5 and 6, is likely to arise from response saturation mechanisms (Nelson and Takahashi 1991, 1999). This refers to a mechanism in which the response of the circadian system is constrained to a maximum possible value. If prior light exposure elicits a partial response, this leaves reduced response potential to subsequent light. This mechanism causes the intensity-response function by prior light exposure to become compressed along the response axis. Nelson and Takahashi (1991, 1999) have demonstrated that this mechanism explains much of the response characteristics of the circadian system to short light pulses (up to 5 min). In a recent mathematical model of the circadian pacemaker (Beersma et al., 2008) it is assumed that the SCN is composed of pacemaker cells that each can respond to light in 2 narrow time intervals, either immediately before its daily interval of electrical activity (leading to advance) or immediately after it (leading to delay). Cells that initiate electrical activity in response to light are assumed to remain active for a couple of hours irrespective of continued exposure. Cells at the end of their activity interval that remain active in response to light are assumed to do that for a while (in the order of a few hours) until exhaustion. If light intensity is not sufficient to trigger a response in a cell, the cell will remain available to respond to subsequent exposure. Since there is a limit to the number of responsive cells, this automatically leads to response saturation.

Essentially, response saturation mechanisms function to achieve similar phase shifts within a short time interval (i.e., in the order of minutes up to 1 h), irrespective of the distribution of light over the interval (Nelson and Takahashi, 1991). Much light in the beginning of the interval will reduce the response to later light; little light in the beginning of the interval will allow for a big response to later light. As a result, such a response pattern will reduce day-to-day fluctuations in pacemaker phase.

The incremental PRCs of Figure 5 for later hours in the pulse are not mutually identical. Additional experiments are needed to test whether or not the differences in shape of the incremental PRCs are due to random fluctuations. According to our model of the SCN (Beersma et al., 2008), however, the beginning of a light pulse is expected to advance those pacemaker cells that are in the appropriate phase of their cycle. The light pulse is also expected to delay those pacemaker cells that happen to have the appropriate phase for delays. In the model these 2 types of responses are not symmetrical. It is likely, depending on phase, that, for instance, more pacemaker cells will respond with advances than with delays. In that case, the 2nd hour of the pulse will be applied to a system in which cells that can respond with delays are relatively overrepresented. This implies that the PRC for the 2nd hour of the pulse can have a different shape compared with the 1st hour, which may even explain the relatively smaller response reduction factor for that hour of the pulse. As argued above, more experiments are needed to substantiate such changes in pacemaker constellation in response to the beginning of a light pulse.

In this study we analyzed data obtained under conditions of exposure to constant light intensity. No data are available on phase shifts under more naturalistic light profiles that include dawn and dusk and the many fluctuations due to the consequences of weather conditions and behavioral modulations of light intensity. The method of measuring PPCs presented here is a straightforward way to determine the consequences of naturalistic light for entrainment of the pacemaker.

ACKNOWLEDGMENT

This work was supported by the EC FP6 integrated project "EUCLOCK" (contract 018741).

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