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Invited Review

Twilight Times: Light and the Circadian System

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Whose twilights were more clear, than our mid-day.
John Donne (1571–1631)
Of the progress of the soul

THE CIRCADIAN CLOCK

The spatial and temporal features of the environment have provided the abiotic selection pressures that have shaped the evolution of life on earth. We are accustomed to accept that the spatial world offers specialized niches. However, we rarely consider that the temporal structure of our planet offers similar opportunities. Most organisms have evolved specializations that allow them to exploit their environment in terms of both space and time, and this demands that individuals have an endogenous representation of these environmental features. We know surprisingly little about the endogenous representation of space, but in recent years an understanding of how organisms build a representation of their temporal world has begun to emerge.

Organisms do not merely respond to their environment, they also have the capacity to adjust physiology and behavior in anticipation of changing environmental conditions. In a competitive world, "being prepared" offers a great selective advantage. It takes considerable time to bring about the complex realignments of physiological systems that permit an optimal expression of different behavioral states (*e.g.* activity and rest or exploitation of temporally restricted resources). By fine-tuning physiology in advance of the changing conditions, an organism can be ready to exploit the changed conditions to its best advantage.

At the heart of the biological machinery that "creates" a day within us is a biological or circadian clock. In mammals, for example, this resides within a small paired nucleus in the brain located above the crossing of the optic nerves, the suprachiasmatic nuclei or SCN.† The neuronal activity of this nucleus continues to oscillate with a 24 h rhythmicity even if it is isolated from the rest of the brain (1). Long before the primary circadian clock of mammals was identified in the SCN, the endogenous nature of circadian rhythms was recognized, first in plants (2–5) and then in many different animal species (6). Identification has been based upon the critical observation that when organisms are kept in isolation, void of any temporal cues, their daily routine continues unabated, although the endogenous daily period (τ) may deviate from the external 24 h cycle (T), hence the term circadian (about 1 day). Such drifting rhythms are often termed "free-running" rhythms, and depending on the organism and on the nature of the constant conditions (*e.g.* constant light or constant darkness), the endogenous period of the free-running rhythm can range from about 19 to 28 h.

The circadian period not only depends on the quality of environmental conditions, as will be discussed below, but is also under tight genetic control as shown by classic as well as by molecular genetics (7). Single genes or gene complexes have been isolated in the fruit fly *Drosophila* (8), the fungus *Neurospora* (9,10), in *Chlamydomonas* (11) and in the cyanobacterium *Synechococcus* (12), as well as in the hamster (13) and the mouse (14), that profoundly influence the length of the circadian period and are likely to be molecular components of the clock itself. The current models for molecular pacemakers will be briefly described in a separate section. Considerable research has shown that the mechanisms of the circadian pacemaker (the endogenous rhythm generator) are part of the biochemistry of single cells (15–19). This has been known for decades because the metabolism of single cell organisms, such as *Euglena* (20,21), *Chlamydomonas* (22), *Acetabularia* (23), *Gonyaulax* (24), *Pyrocystis* (25), *Tetrahymena* (26) and *Paramecium* (27) is also controlled by a circadian system (for an extensive review of circadian rhythms in unicellular organisms see Edmunds (28)).

In addition to the free-running nature of circadian rhythms, two other universal properties have been identified: (a) The period of circadian rhythms is not greatly affected by changes in environmental temperature. The rates of most biochemical reactions approximately double with a 10°C rise in temperature ($Q_{10} \geq 2$). By contrast, circadian rhythms show temperature compensation so that period length changes very little over larger temperature fluctuations (29). As a result, those circadian rhythms studied have a Q_{10} that

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†*Abbreviations:* CT, circadian time; PRC, phase response curve; RGC, retinal ganglion cells; RHT, retino-hypothalamic tract; SCN, suprachiasmatic nuclei.

is very close to 1. (b) Circadian rhythms can be adjusted so that internal and environmental time are synchronized. It is this process of entrainment that forms the central focus of our discussion below.

ENTRAINMENT OF CIRCADIAN RHYTHMS

The function of the circadian system is to regulate the phases at which biological events occur, either in relation to specific features of the 24 h environmental cycle or in relation to periodic events within the organism. To fulfill its function, the circadian system must remain synchronized with the solar day. This entrainment is accomplished by resetting mechanisms that correct for the deviations of the endogenous period from 24 h ($T - \tau$). These consist of an input pathway (receptor and transduction elements) for specific environmental signals (zeitgeber) and of elements within the circadian pacemaker, capable of transforming the incoming signals to appropriate changes of the rhythm's phase. When entrained, the circadian clock adopts a distinct phase relationship with the astronomical day, and each of the different expressed rhythms adopts its own phase relationships with the clock.

Circadian systems respond, depending on the species and its spatial and temporal niches, to a variety of different zeitgeber. For example, many microorganisms, plants and heterothermic animals can be entrained by regular changes in the ambient temperature (28). Social signals like species-specific songs in birds (30) or sound signals in humans (31) can act as zeitgeber, and in some species regular feeding schedules can also result in entrainment (32,33). However, the stable and systematic daily change in the quality of light at dawn or dusk provides the most reliable indicator of the phase of the day. As a result, most organisms have evolved to use the twilight transition as their primary zeitgeber to adjust circadian phase (photoentrainment). Note that most of the regular daily changes in other physical properties (temperature, humidity, *etc.*) or biological changes (availability of resources or the danger by predators) are in some way linked to the solar day and can thus be predicted by using light as the zeitgeber. Regardless how short or long the circadian period may be in constant experimental conditions, in nature the discrepancy between τ and T will be relatively small and will be corrected for in the twilight zones, either at dawn (if $\tau > T$) or at dusk (if $\tau < T$), by advancing or delaying respectively.

Features of the photic environment

Before we consider the mechanisms by which circadian systems use light as a zeitgeber or some examples of photopigments, receptors and transduction pathways that are involved, we need to consider what features of twilight are important for photoentrainment. During twilight the quality of light changes in three important features. There are large changes in the amount of light, its spectral composition and in the sun's position relative to the horizon. All three features could be used by organisms for detecting the phase of twilight.

The amount of light. On a minute to minute timescale, there can be huge changes in the light environment. Cloud cover, moving and shadowing or directly looking at the sky can greatly alter the amount of light detected by an organism. As a result, the sensory system needs to smooth out these local fluctuations to obtain a reliable measure of light levels, and hence time of day. One way to achieve this would be to use a long sampling or integration time to gather photons. In addition, the system would need to measure overall light levels in the environment (irradiance) and ignore brightness in particular areas of the sky (radiance).

The spectral composition of the light. In addition to profound changes in irradiance at twilight (approx. 6 log units), there are very precise spectral changes; twilight is primarily characterized by relative enrichment of the shorter wavelengths (<500 nm) compared to the mid–long wavelengths (500–650 nm). If circadian systems used two photoreceptors (with different spectral sensitivities) to sample the relative amounts of short- and longwave radiation, then this could provide a very reliable marker of the phase of twilight.

The position of the sun. The position of the sun relative to the horizon, could also provide a very accurate measure of the phase of twilight. Of course, the sensory requirements for plotting the sun are very different from the requirements for measuring the overall levels of light in the sky. Mapping the position of the sun would require an elaborate radiance detector, possibly with a lens, and the maintenance of topographic order between the photoreceptors and the site of photic integration.

The extent to which all the features of twilight are encountered by an organism will depend upon its ecology and strategy for sampling light.

The ecology of photoentrainment

Organisms are often highly adapted to both a spatial and temporal niche (34). The most obvious example for temporal specialization is the timing of activity to portions of the light (day active), dark (night active) or twilight zones (crepuscular). Occupation of these temporal niches has resulted in distinctive specializations. For example, night-active animals rely more on their olfactory, acoustic or mechanosensory systems, while day-active ones primarily use the visual sense. The opening of flowers, the eclosion of insects or the time of birth are other examples of functions that are restricted to unique times of the day characterized by specific environmental properties (light intensity, humidity, wind, the presence of other organisms such as predators or pollen-collecting insects, *etc.*).

Due to the high degree of temporal adaptation of each species, activity outside these specialized niches is costly. The twilight zones are the times when predators are most successful in catching both day- and night-active animals. Thus, most animal species try to avoid being openly active during the twilight zones. The specific timing of behavior determines a distinct pattern of exposure to natural light (light sampling behavior). The diagrams in Fig. 1 are, of course, highly schematic and do not reflect the considerable "noise" in the light fluence due to behavior, clouds, moon phases, time of year, *etc.* Generally, day-active animals as well as plants are exposed to long, continuous stretches of darkness and light. Light exposure is different for burrowing and nonburrowing animals (compare A and B; C and D–F in Fig. 1). Night-active animals are rarely exposed to full

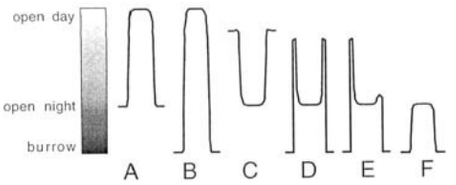

Figure 1. Different light sampling due to specific behavior (light intensities are indicated by the gradient on the left). Each curve in the diagram represents the light intensity to which organisms are exposed or expose themselves. They are all centered around the midpoint of the activity, A,B around noon and C,F around midnight. (A) Day-active, nonburrowing animals (*e.g.* cattle) and plants; (B) Day-active, burrowing animals (*e.g.* ground squirrels); (C) night-active, nonburrowing animals (*e.g.* some owls); (D) night-active, burrowing animals (*e.g.* most rodents and many bat species); (E) same as D, but taking into account that the phase of activity is not symmetrical to dawn and dusk; the phase position of the activity can also change for one species over the course of the year; (F) night-active, burrowing animals that begin their activity after dusk and end it before dawn (*e.g.* some bats living in dark caves); note that the relationship between activity and light exposure is very similar to the one of day-active organisms. Redrawn after Kenagy (36).

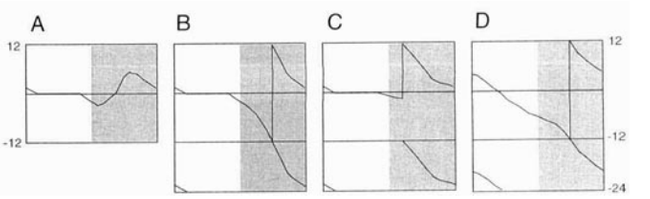

Figure 2. Different types of phase response curves (PRC) for incremental light pulses. When single light pulses are given at different times of the cycle (abscissae) then rhythms are either advanced (positive ordinates) or delayed (negative ordinates). (A) Typical type 1 PRC (*e.g. Drosophila melanogaster* or hamster); (B) typical type 0 PRC (*e.g. Drosophila pseudoobscura* or *Bryophyllum*); (C) asymmetrical type 0 PRC (*e.g. Gonyaulax*); (D) type 0 PRC without dead zone (*e.g. Neurospora*).

daylight intensities, but some species (*e.g.* barn owls) spend the day in covers that are still exposed to considerable amounts of light (Fig. 1C). Most night-active animals spend the day in more or less light-tight burrows. Depending on species, but also on time of year and latitude (35), these animals can sample light very specifically either at dawn, dusk or both (Fig. 1D and E). Finally, some night-active species are exposed to the same temporal light structure as day-active animals, although at different intensity levels (compare Fig 1B and F).

The phase response curve to light

An essential feature of the resetting mechanism, to ensure stable entrainment, is that the induced phase changes caused by the zeitgeber vary systematically throughout the endogenous cycle. This characteristic can be drawn as a phase response curve (PRC). In the experiments determining PRC, single light signals (either as increasing or decreasing steps of the light level or as short pulses of light or darkness) are given at different times of the circadian cycle to organisms that are kept in otherwise constant conditions. The PRC for incremental light pulses have been measured in many different microorganisms, plants and animals and are remarkably similar. When light pulses are given during the subjective day (the time of the cycle that corresponds to the light phase under entrained conditions) the phase of most circadian systems is not changed, this is called the "dead zone" of a PRC. When pulses are presented in the late subjective day or the early subjective night, the phase is generally delayed (activity starts later the next cycle) while it is advanced (activity starts earlier) when the pulse is given at other times during the subjective night or around dawn (Fig. 2A). By definition, advances are drawn as positive and delays as negative phase shifts; the transition between delays and advances is called the PRC "breakpoint." Although the general shape of the light pulse PRC is similar for all circadian systems, there may be big differences in the amplitude of the phase shifts. Low amplitude PRC, like the one shown in Fig. 2A, are called type 1, while those that reach phase shifts of 12 h or more are called type 0 (for a detailed definition of the two PRC types, see Winfree (37) and Pittendrigh (38). In type 0 PRC (*e.g.* Fig. 2B), the break point becomes somewhat arbitrary because a 12 h delay and a 12 h advance both represent the same phase shift by half a circadian cycle. Therefore, phase shifts of type 0 PRC can be plotted on a continuous line (as shown in Fig. 2B and D). The position of the break point can be specific for a given PRC (compare Fig. 2B and C), and the extent of the dead zone can also vary between organisms and may not even exist (compare Fig. 2B and D). In some systems, however, the advances show a typical type 0 response with phase shifts up to 12 h or more, while delays are much smaller (Fig. 2C). These asymmetrical PRC indicate that delays and advances may be caused by different mechanisms, and an example for this type of PRC in a marine alga will be discussed in detail.

In addition to its entraining effects, light can modify both the period and expression of circadian rhythms. In most species, period can be substantially altered upon exposure to constant light (Aschoff's Rule) (39). Some (predominantly day-active organisms) have a period that gets shorter the higher the light intensity, whereas others (predominantly night-active organisms) show the opposite period–intensity relationship. For example, nocturnal mice exposed to constant bright light will increase their circadian periods by 2–3 h (Fig. 3). In addition, many species will gradually become arhythmic in constant light. This is an important observation because it complicates the interpretation of experiments that have attempted to identify genes that form part of the circadian pacemaker. For example, a recent screen of mutagenized mice identified a long-period circadian phenotype (*clock*) (14,40,41). In constant darkness, homozygous *clock* mice show a period between 27 and 28 h for several cycles before becoming arhythmic. Although both the persistence and period of the circadian rhythm have been affected by *clock,* no assumptions can be made about the function of the defective gene on the basis of these observations alone. It is possible that the *clock* phenotype has resulted from a defect in a gene that mimics the effects of constant bright light on the clock, rather than a defect in the clock itself.

The regime by which light synchronizes the circadian system is still not fully understood. It is clear that the light PRC is an essential prerequisite for entrainment. Light PRC are generally constructed with the help of singular light pulses; however, the amount and the duration of the light pulse necessary to shift the circadian phase varies from species to

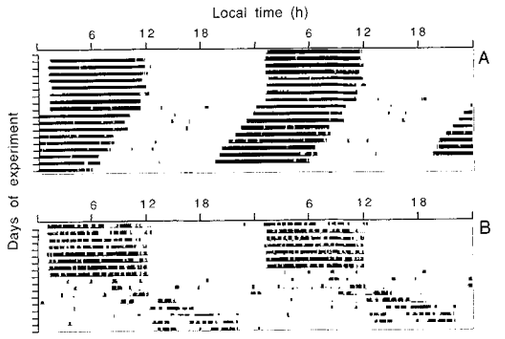

Figure 3. Double-plotted wheel-running activity record for two mice under different lighting conditions. Note that 24 h records are often double-plotted in this form, so that the rhythmic pattern can be more easily followed across "midnight." (A) Shows 17 days of individual wheel-running activity. For the first 7 days, the mouse was maintained on a light : dark cycle of 12 h light and 12 h dark (LD 12:12), which entrained its activity. At the end of day 7, the LD cycle was terminated and the mouse did not experience the expected "dawn" on day 8, remaining in constant darkness (DD) for the rest of the record. The free-running period (τ) in DD was approximately 23.5 h. (B) In this example, the mouse was exposed to LD 12:12 for 8 days. At 02:00 on day 9, the mouse was exposed to constant light (LL) at an irradiance of 820 mW cm^{-2} (1700 lux) using a fluorescent light source. At this irradiance of LL, τ was approximately 25 h. Brighter LL will increase t even further. Note that levels of activity are also reduced under LL conditions (Lupi and Foster, unpublished).

species. For each species, there is an optimal range of time over which photon flux can be integrated to maintain sensitivity (reciprocity). For example, the circadian system of the golden hamster is maximally sensitive to 1×10^{11} photons (λ_{max} 506 nm) delivered during a 5 min light exposure. When the same number of photons is delivered for different durations of time, the hamster was found to be relatively insensitive to stimulus durations of less than 30 s, but intensity : duration reciprocity was maintained for stimuli up to 45 min (42). Note that this reciprocity in the circadian system far exceeds that of classical visual responses that are typically limited to milliseconds or seconds.

The hamster is a nocturnal, burrowing animal sampling the light environment at dawn and dusk (Fig. 1D). One can easily envisage how a light PRC is utilized by such an animal, but it is more difficult to understand how a light PRC is used by a day-active animal exposed to continuous light of 12 h or more. Is the circadian system in this case set only once or twice per day when light intensities reach a certain fluence threshold (discrete entrainment) or is it continuously reset (or modulated) by light throughout the photoperiod (continuous entrainment)? To answer these questions, one has to find out which part of a light pulse is responsible for the resulting phase shift. What is the contribution of the onset of the light pulse, the level and/or duration of the light or the termination of the pulse? The large number of possibilities has not encouraged experimental analysis. In one of the few studies undertaken, however, a comparison of the effects of step-up and step-down PRC found that these stimuli lead to different forms of PRC (43).

The situation is made even more complex by dark-pulse PRC. When the conditions of the protocol for measuring light-pulse PRC are inverted, *i.e.* when organisms are kept in constant light and exposed to single pulses of darkness, the shape of the resulting dark-pulse PRC is often not symmetrical to the light-pulse PRC. Compared to the large number of light-pulse PRC, very few experiments have been done with dark pulses. Dark-pulse PRC are typically of type 1 (*i.e.* show only smaller phase shifts) and rarely contain significant dead zones. Maximum advances are usually found at the end and maximum delays at the beginning of the subjective day. For a comprehensive collection of PRC, see the PRC atlas, compiled by Carl H. Johnson (Vanderbilt University).

In spite of these unsolved questions, it is evident that the general shape of the light PRC is a good predictor for the effect of light at different times of the cycle. Light around dusk will set the oscillation back or slow it down, while light around dawn will set it forward or speed it up. It becomes clear from Fig. 1 that the entrainment regime will depend very much on the species' light sampling behavior. An extreme example for this specificity is the flying squirrel; these animals rest in a light-tight tree burrow during the day, become active after dusk and return before dawn. As a result of this behavior, their circadian system freeruns in almost constant darkness for several days with a period shorter than 24 h until the onset of activity "bumps" into dusk, and the animals expose themselves to light at a phase of their PRC that delays the rhythm. After this reset, the sequence of events starts all over again (44). In addition to ensuring that $\tau = T$, the PRC can partially explain how behavior can be aligned to an expanding and contracting photoperiod in the nonequatorial latitudes. For example, a nocturnal rodent emerging from its burrow at dusk and encountering light will be phase delayed the following day and leave its burrow later. In the same way, if the rodent encounters light before it returns to its burrow at dawn, then its clock will be phase advanced and stimulate activity to start earlier the following day. In this way, the PRC can "nudge" behavior back and forth so that activity can be confined to that part of the light environment that promotes survival. Of course, light acting on the PRC is not the only way in which behavior can be adjusted; light can directly excite or inhibit activity in many organisms. In nocturnal rodents, for example, bright light delivered during the night will suppress activity (negative masking) (45), whereas dim light may even increase levels of activity (positive masking) (Mrosovsky and Foster, in preparation). As a result, the expressed rhythm of an organism is often the product of both circadian and masking influences.

All light PRC share similar features; the largest phase shifts occur during the subjective night, with phase delays around dusk and advances around dawn. These features argue that the twilight zones were the main driving force shaping the phase response characteristics of all circadian systems, irrespective of the temporal niche (*e.g.* day or night active) and independent of the rhythmic function within an organism (*e.g.* eclosion and activity in the fruitfly). During both twilight zones all light qualities change – radiance, irradiance and spectral quality – and the extent and strategies by which circadian systems of different organisms make use of these changes will be now considered.

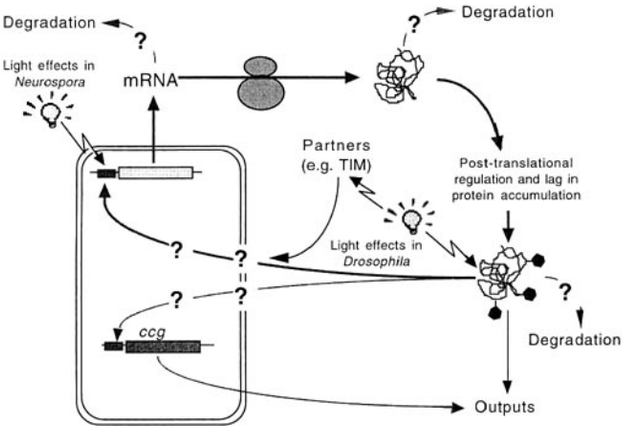

Figure 4. Molecular model of a circadian pacemaker (CCG: clock-controlled genes; see text for further details).

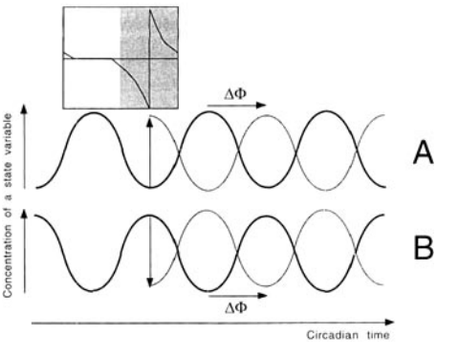

Figure 5. The effect of light pulses on a state variable of the molecular oscillator. (A) Light increases the concentration of the variable; (B) light decreases the concentration of the variable (see text for details). If light either increases the state variable to its maximum level or decreases it to its minimum, the resulting phase shifts give rise to a typical type 0 PRC.

LIGHT AND THE CIRCADIAN SYSTEM

For spatial orientation, many organisms use photoreception, which is also used for temporal "orientation," *i.e.* for entrainment by light. However, spatial and temporal orientation apparently use different photoreceptors and transduction pathways (in some species even different sensory organs, such as eye and pineal). An independent light input for spatial and temporal orientation has not only been found both in unicellular organisms and in higher animals as will be described in the following sections. In these, we will mainly concentrate on two phylogenetically very diverse examples, the marine dinoflagellate *Gonyaulax polyedra* and the mouse. In other phyla, such as higher plants (46), insects (47), molluscs (48), reptiles and birds (49,50) the effect of light on the circadian system will be similar, as described above, but the respective receptors and transduction pathways may be very different and surely will have adapted to the specific spatial and temporal environment of the respective organism.

Research into the mechanisms of photic entrainment has to address at least three basic questions. Which pigments and light receptors are involved? What are the elements of the transduction pathway? And finally, how does the transduced light signal eventually act on components of the circadian oscillator? These questions can be asked at the molecular level, at the level of single cells and at the systemic level in multicellular organisms. In multicellular animals and plants, the receptor elements may be separated from the organ that is responsible for the generation of the circadian rhythmicity (*e.g.* the retina and SCN of mammals), or receptor and clock may be within the same cell (*e.g.* the pinealocytes of some birds and reptiles). In unicellular organisms, photoreceptor, transduction cascade and oscillator are always part of the same cell.

Light and the circadian system at the molecular and cellular level

The last decade of circadian research has greatly increased our knowledge about the possible cellular and molecular mechanisms that are responsible for the endogenous generation of a 24 h rhythmicity (51–55). This research has mainly been conducted using the model systems in *Drosophila* (8) and the fungus *Neurospora* (9,10) and more recently also in the mouse (14,56), cyanobacteria (12,57) and higher plants (58). The results give rise to the following molecular model (Fig. 4).

The circadian oscillation is basically generated by an autoregulative negative feedback loop involving at least one pacemaker gene. This gene gives rise to a message (mRNA) and a protein that both cycle in a circadian fashion and are considered so-called "state variables" of the molecular oscillation. The clock protein either acts on or is a transcription factor, down-regulating or inhibiting its own gene expression. Several important features of this feedback loop still have to be worked out in detail: (1) What parts of the feedback loop contribute to the necessary delays adding up to the relatively long circadian periodicity? (2) How are post-translational modifications of the protein involved in the rhythmicity? (3) What are the circadian mechanisms of gating the nuclear entry of protein(s)? (4) What is the nature of the kinetics of degradation of the different components (these are crucial because they determine whether the system oscillates or not)? (5) Which and how many partner factors are involved? (6) Finally, how do state variables control the circadian outputs?

Other properties of the circadian system such as temperature compensation and entrainment by light have also been investigated at the level of the molecular oscillator. Regardless of how light is absorbed and transduced, it finally will have to affect one or more of the molecular state variables to elicit a phase shift. There are at least two theoretical possibilities for this effect. Light can either increase (Fig. 5A) or decrease (Fig. 5B) the concentration of a state variable. To elicit, for example, a 12 h phase shift in the middle of the subjective night in both of the two possibilities, the respective state variables have to oscillate 180° out of phase. In case of increasing the concentration, the maximum of the state variable coincides with the dead zone of the PRC, while it is the minimum in the other example.

It has been shown experimentally that these two possibilities do indeed exist (see also Fig. 4). The *frq* and *per* mRNA levels in *Neurospora* and *Drosophila* oscillate approximately 180° out of phase. In *Neurospora,* light appears to shift the

phase by rapidly increasing *frq* mRNA (59), which is low during the subjective night, while in *Drosophila,* light affects posttranslational modifications of the PER protein (60), decreases the level of PER-TIM dimerization and moderates the overall amount of TIM. The TIM is a partner protein in the *Drosophila* model that appears to be necessary for the nuclear entry of PER (see also Fig. 4) (60–65). A recent study has also shown that the effects of light on PER and TIM phosphorylation and on the two proteins forming a complex are not restricted to light pulses but that these processes are similarly affected by constant light (66). In addition, this study shows that the interactions between light, PER and TIM differ among the various *per* mutants in a way that is consistent with the observed physiology of the respective phenotypes. These observations also offer a molecular explanation why *Drosophila* (and *Neurospora*) become arhythmic under constant light.

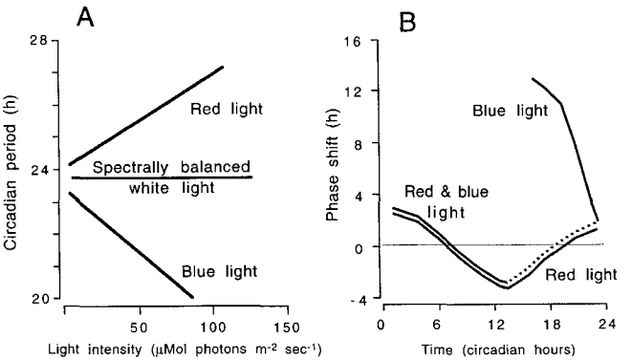

Figure 6. Spectral effect on the circadian system of *Gonyaulax.* (A) The period–intensity relationship depends on the spectral composition of the constant light; redrawn after Roenneberg *et al.* (80) and Roenneberg (81). (B) The light pulse PRC is different for red and blue light pulses; redrawn after Roenneberg and Deng (79).

Light and the circadian system in a unicellular alga

In the freshwater alga, *Chlamydomonas,* phototaxis is mediated by a rhodopsin (67,68) and, depending on the level of illumination, two other photoreceptors elicit the circadian light responses. One of them, possibly a chlorophyll judged by its absorption, is active when cells are kept in light (69), another still unknown pigment, with two prominent absorption peaks at 520 and 660 nm, is responsible for resetting the circadian phase when cells are kept in darkness (70). Another example of such complex light perception in circadian systems is the unicellular marine dinoflagellate *G. polyedra.*

The *Gonyaulax* circadian system has been extensively studied at the organismic, the cellular, and the molecular level (19,71), and its mechanisms can be clearly placed relative to their temporal biology. Like many other phytoplankton, these alga migrate vertically over a great depth (some species sink to the bottom as deep as 35 m every day; J. Brenner, personal communication). Both the rise and the decline anticipate the actual light changes of dawn and dusk. During the day, *Gonyaulax* cells form dense swarms (aggregations) at the surface, where they photosynthesize, while the cells take advantage of the higher nutrient concentrations at greater depth during the night. As will be described, this daily temporal behavior is controlled by a complex circadian system that consists of two circadian oscillators each controlling different output rhythms and that receives light *via* two light input systems. Although this will not be discussed in detail here, it should be mentioned that recent experiments have shown both light and nutrients act as zeitgeber on the *Gonyaulax* circadian system and that both photic and nonphotic zeitgeber show strong interactions (72).

Figure 1 shows the specific light-sampling behavior of different species; however, only light fluences are taken into account in this diagram. Due to the preferential light absorption of sea water (73), *Gonyaulax* cells migrate both through intensity and spectral gradients, and their circadian system actually takes advantage of this spectral information. As in *Chlamydomonas,* the *Gonyaulax* circadian system responds to light both in the blue and the red part of the spectrum (475 and 650 nm). This has been determined with the help of an action spectrum for phase shifting the algae's bioluminescence rhythm by light pulses (cells were kept in constant darkness except for the light pulse) (74). When biological functions in photosynthesizing organisms are controlled both by red and blue light, it generally points to the involvement of either a phytochrome or of the photosynthetic machinery. The presence of phytochrome has so far not been found in *Gonyaulax,* but experiments with the photosynthesis inhibitor DCMU (N′-3,4-dichloro-phenyl-N,N-dimethyl urea) indicate that a chlorophyll may be partly responsible for circadian light responses (75,76). Action spectra are a necessary basis for finding the pigment(s) responsible for a specific photoresponse, but they do not necessarily answer the question of how many photoreceptors are involved. The first indication for the involvement of more than one photoreceptor in the *Gonyaulax* circadian system originates in experiments where the period's dependence on the light's fluence rate (period–intensity relationship) was measured under different spectral light conditions (77). Increasing fluence rates of short wavelength light shorten the period, while an increase of red light lengthens it (Fig. 6A). These two opposite effects are additive and can be "titrated" by presenting both parts of the light spectrum at comparable fluence rates; increasing fluence rates of incandescent light (containing both short and long wavelengths) or a mixture of separate red and blue light sources do not significantly alter the circadian period.

The sustained effects of different light intensities on the period in constant conditions and the acute effects of light pulses on phase shifting have been shown to be related (velocity response hypothesis (78)); period–intensity relationships of circadian systems can be predicted from the shape of their light PRC: the larger the advance portion of a PRC the more the period will shorten when the intensity of the constant light is increased. The different period–intensity relationships for red and blue light in *Gonyaulax,* therefore, predict different PRC for the respective light pulses. This prediction has been supported experimentally. When red or blue light pulses are given during the subjective day and the first few hours of the subjective night (before circadian time [CT] 15), both light pulse types elicit identical phase responses (Fig. 6B). However, when the two light pulse types are given during the remaining part

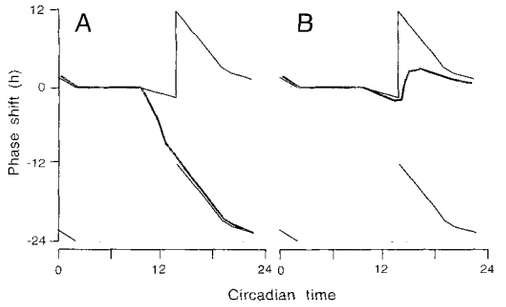

Figure 7. The effect of chemicals on the light PRC in *Gonyaulax*: (A) creatine, redrawn after Roenneberg and Taylor (76); (B) allopurinol, redrawn after Deng and Roenneberg (82).

of the subjective night, blue pulses greatly advance the rhythm while red pulses still lead to considerable delays (79). One of the two receptors is mainly blue sensitive, strongly advancing the system in the subjective night; the other is both red and blue sensitive, mostly delaying the rhythm throughout the subjective day. Thus, the period–intensity relationship and the PRC in *Gonyaulax* are entirely consistent with the velocity response hypothesis. The PRC of the red/blue input is of type 1 and cannot be changed into a type 0 PRC even at high fluence rates of the pulse (>100 μmol photons m^{-2} s^{-1}), while the former is highly light sensitive, leading to phase shifts of 12 h or more already at 5 μmol photons m^{-2} s^{-1}. Besides proving the existence of the postulated two light inputs, the results on phase shifting reveal another important property of the circadian light input in *Gonyaulax,* namely that the blue-sensitive input appears to be itself under circadian control.

The shape of the *Gonyaulax* light PRC is somewhat exceptional because it does not show the continuity of other known type 0 PRC (Fig. 2). This property can, however, be explained in view of the results described above. The blue-sensitive input appears to be activated only after CT 15; at earlier CT, the red/blue system is responsible for the observed phase shifts that mainly consist of phase delays. The red/blue system can adapt to red light, so phase shifts by light reaching the pacemaker through this input pathway are greatly attenuated when constant red light is used as background light (in *Gonyaulax,* most light PRC are measured in constant red light and the delays before CT 15 are often smaller than shown in Fig. 2C or even absent).

Experiments with chemicals specifically affecting the blue-sensitive system have supported the hypothesis that the PRC discontinuity is due to properties of the light input pathway and not due to properties of the pacemaker itself. The mammalian phosphagen creatine shortens the circadian period in constant white or blue light but not in red light (80). This shortening in constant light can be explained with the effects of creatine on phase shifting with pulses of short wavelength light. Creatine appears to advance the activation of the blue light input to an earlier circadian phase (76), thereby "filling in" the discontinuity of the light PRC (Fig. 7A). This shows that the pacemaker's state variables in *Gonyaulax* indeed show a generic type 0 resetting behavior as would be predicted by the model shown in Fig. 5. The discontinuity of the light PRC must, therefore, be due to the circadian feedback on the blue light-sensitive transduction pathway, which is apparently not active before CT 15.

Another chemical that has proven itself useful in the dissection of the light input to the *Gonyaulax* circadian system is allopurinol, an inhibitor of xanthine oxidase. Allopurinol specifically inhibits the effects of white and blue light pulses given after CT 15, while it does not change the effect of red or blue pulses given before CT 15 (Fig. 7B) (82). Again, allopurinol not only affects phase shifting by light pulses but also lengthens the period specifically in blue or white light. By inhibiting the blue-sensitive light input, it can even reverse the period–intensity relationship for short-wavelength light to the one normally recorded in constant red light (see Fig. 6A).

But what do these pharmacological experiments tell us about the transduction pathway of light to the circadian pacemaker? The molecular mechanisms of light effects on the *Gonyaulax* circadian system are still not known. They apparently involve protein phosphorylation because the kinase inhibitor DMAP (6-dimethylaminopurine) blocks the effect of light pulses at any circadian phase (83). This effect acts probably more downstream in the light transduction compared to the input-specific effects of creatine and allopurinol, possibly acting on a state variable that is the final target of both light input pathways. How creatine acts biochemically in *Gonyaulax* is still enigmatic. In mammals, creatine acts as a transport molecule for high-energy phosphate groups; its role or even its existence in plants is unknown, and we found no evidence for its presence in *Gonyaulax* (84). In contrast, xanthine oxidase activity can actually be measured in *Gonyaulax* extracts and is under circadian control, showing a 15-fold difference over the course of one cycle (Deng and Roenneberg, unpublished results). We had originally used allopurinol in a series of experiments testing the influence of inhibitors of pigment synthesis and of flavoproteins (82). Whether the flavoprotein xanthine oxidase is directly or indirectly involved in the light transduction mechanism is still an open question.

The complexity of the *Gonyaulax* circadian system is still increased by the fact that it contains at least two circadian oscillators (85). One of these (the B-oscillator) controls the bioluminescence rhythm and possibly also vertical migration; the other (the A-oscillator) controls the cells' swarming behavior (aggregation), which involves phototactic orientation (86,87) and possibly also controls photosynthesis (81). The two oscillators respond differently to light signals; while the B-oscillator is mainly blue sensitive, the A-oscillator also responds to changes in long-wavelength light (88). The insights into the circadian system of this marine alga show the complexity as well as the adaptivity of temporal programs (71). Circadian light input can respond to both fluence and to spectral changes, and different output rhythms are selectively controlled by different circadian oscillators. In addition, light responses and the phase of different output rhythms in relation to each other as well as in relation to the environmental cycle are affected by the availability of nutrients (72).

Light and the circadian system of mammals

Unlike the other vertebrate classes, loss of the eyes blocks all circadian responses to light in every mammal examined

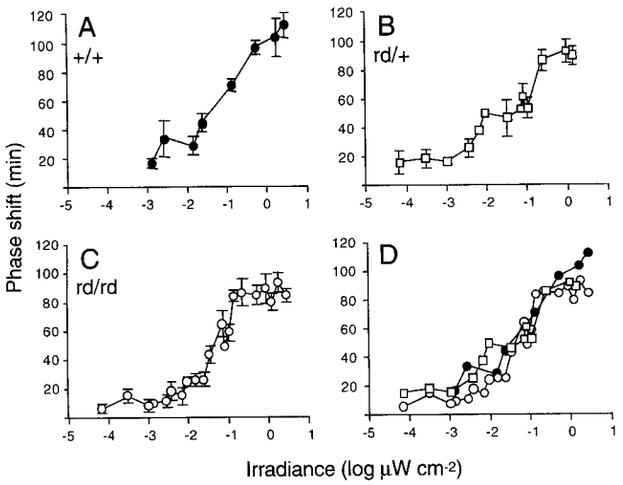

Figure 8. The magnitude of delaying phase shifts produced by a 15 min light pulse as a function of irradiance in mice with (A) normal retinas (+/+); (B) normal retinas (*rd*/+); (C) degenerate retinas (*rd/rd*). For comparison the three irradiance response curves (A–C) have been superimposed in D. Despite the massive damage to the visual photoreceptors and loss of visual function in *rd/rd* mice, these animals show circadian responses to light that are indistinguishable from those of mice with normal retinas. Error bars ± SEM (redrawn from Foster *et al.* (90)).

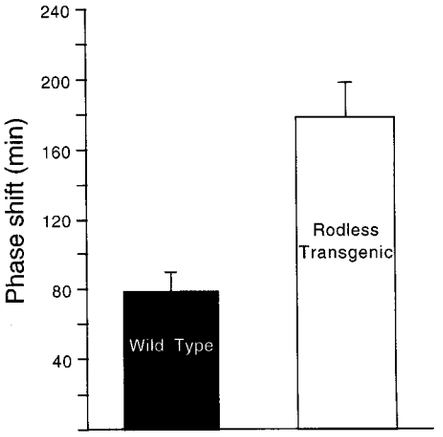

Figure 9. Histogram showing the mean phase delay of congenic wild-type and rodless transgenic mice (*rdta*) in response to a single 15 min light pulse at saturating irradiances (7 mW cm^{-2}; 510 nm). Phase shifts in *rdta* mice are approximately 2.5× greater than congenic wild-type mice. Sample sizes are indicated in parentheses. Error bars represent SEM (Lupi, McCall and Foster, in preparation).

(89,90). This is the basis for stating that the entraining photoreceptors of mammals must be ocular. But in reality, examination of photoentrainment and circadian organization has been restricted to a limited numbers of species in a few orders of eutherian mammals. Most reviews of mammalian physiology are usually reviews of rodent physiology, and the discussion below is unfortunately no exception. Before we examine the entraining photoreceptors in the eye, there should be some mention of the pineal, which in most non-mammals is a photoreceptor organ. Mammalian pinealocytes show some ultrastructural features usually associated with photoreceptors (91) and contain a range of photoreceptor proteins including opsin (92,93). However, the mammalian pineal contains no chromophore (11-*cis*- or all-*trans*-retinaldehyde). These results are in marked contrast to the findings in the non-mammalian vertebrates, where 11-*cis*- and all-*trans*-retinoid have been isolated from the pineal (94,95). All the evidence suggests that the opsins present within the mammalian pineal appear not to be part of a functional photopigment and that the pineal plays no role as a photoreceptor for entrainment of circadian rhythms (92,96,97).

In an attempt to define the role of rods, cones and other retinal neurons in entrainment, retinally degenerate mice have been employed as reduced preparations, correlating the loss of retinal elements with loss of entrainment. The first experiments in this area used mice homozygous for retinal degeneration (*rd/rd*). These mice experience a massive degeneration of the rods and cones. By 60 days of age, all rod cells have degenerated, and between 90 and 150 days of age even the crudest electrophysiological and behavioral responses to bright light have disappeared (98). Despite the loss of all rods and most of the cones in *rd/rd* mice, these animals showed circadian responses to light that were indistinguishable from mice with phenotypically normal retinas (*rd*/+, +/+) (Fig. 8) (90).

These results demonstrate that the responsiveness of the circadian system to light appears independent of the loss of rods and cones. Yet, the circadian light input must still be retinal because enucleation of mice (including *rd/rd*) blocks all photoentrainment. The independence of the entrainment pathway from the rod- and cone-mediated visual sense is further substantiated by the investigation of light-induced expression of the immediate early gene c-*fos* within the SCN. Studies from a range of laboratories have demonstrated that c-*fos* is induced within the SCN upon retinal illumination at times when the circadian system is sensitive to light. In addition, levels of c-*fos* expression appear to parallel the strength of the photoentrainment signal. Light stimuli that result in a large phase shift also induce large levels of c-*fos* expression within the SCN (99,100). Experiments using the *rd/rd* mouse have shown that the distribution, number and density of Fos immunostained cell nuclei within the SCN were identical in *rd/rd* and +/+ mice (101).

Circadian responses to light have also been investigated in mice (*rdta*), the rods of which have been "removed" during ontogeny using a molecular trick: a fusion gene was integrated into their genome consisting of a 1 kb fragment of the human rhodopsin promoter linked to an attenuated diphtheria toxin gene. As a result, rod cells were "poisoned" when the cells started to express rhodopsin. Morphological, physiological and molecular analysis of these retinae have demonstrated that rod degeneration begins soon after the onset of rhodopsin expression (around postnatal day 6) and that rhodopsin expression is reduced by approximately 80% by postnatal day 28 (102). Cone cell bodies (with no outer segments), as well as cone opsins, however, persist until at least postnatal day 150. Experiments with light pulses produced a surprising result. At irradiances that produce saturating phase shifts in wild-type mice, congenic *rdta* mice showed shifts approximately 2.5-fold greater (Fig. 9) (103). A comparison of the irradiance response curves for *rdta* and wild-type controls suggests that it is the amplitude

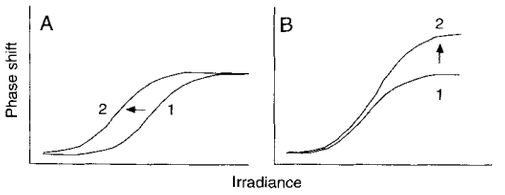

Figure 10. Diagrams illustrating: (A) an increase in the sensitivity of the circadian system to light. The irradiance response curve (1) has moved to the left (2). In this case fewer photons are required to saturate the response. (B) An increase in the amplitude of circadian responses to light. The same number of photons produce a much greater phase shift, but saturation levels in (1) and (2) occur at the same photon flux. Rod loss in *rdta* mice appears to have resulted in a change in the amplitude of clock responses to light.

of clock responses that has been affected in *rdta* mice and not the sensitivity of the clock to light (Lupi, McCall and Foster, in preparation) (Fig. 10).

Note that circadian photosensitivities in *rd/rd* mice were statistically indistinguishable from +/+ mice even at saturating intensities (Fig. 8). So how do we explain the large increase in the amplitude of circadian responses to light in rodless *rdta* mice when compared to rodless *rd* mice? Perhaps the differences between *rdta* and *rd* mice are due to the timing of rod loss during development. At the age tested (80 days) the retinae of *rdta* and *rd* mice appear very similar. Both lack rods and possess few cones. However, the onset of rod ablation in *rdta* mice occurs approximately 1 week earlier than in *rd* mice (102). We suggest that the earlier loss of rods in the *rdta* retina may occur when the retina and/or its central projections are sufficiently plastic to allow significant reorganization. An altered signal to the developing clock may permanently alter the amplitude of clock responses to light. On the basis of the work on *rd* and *rdta* mice rod photoreceptors are clearly not required for photoentrainment, although their early loss may affect the development of the circadian system.

Until the studies on *rd* and *rdta* mice, it had been assumed that rods regulate circadian responses to light in mammals. This belief was based upon an action spectrum for phase shifting of locomotor activity rhythms in the golden hamster (*Mesocricetus auratus*) (104). These data show a spectral maximum (λ_{max}) around 500 nm that resembles closely the absorbance maximum (l_{max} = 502 nm) of the extractable rod photopigment in this species. More recent studies in rodents, however, have demonstrated the existence of cones with spectral sensitivities near 500 nm (105), including the hamsters, which have a cone with a λ_{max} between 505 and 506 nm (106). Because the action spectrum for phase-shifting locomotor rhythms cannot resolve differences between sensitivities at 502 nm (rod) or 506 nm (cone), the roles of rods and/or cones in this species remains ambiguous.

In the normal mouse retina, two types of cones have been identified, a green-sensitive cone with a λ_{max} near 510 nm and a UV-sensitive cone with a λ_{max} near 360 nm (105). Action spectra for phase shifting in aged (80–90 days) *rd/rd* (rodless) and +/+ mice were identical and suggested, as in the case of unicells described above, two spectral maxima. In the mouse, they were found to be close to the spectral sensitivities of the green and UV cones (107). On the basis of this similarity, cones became strong candidates for circadian regulation. However it is worth stressing that although cone opsins and cell bodies remain in the *rd/rd* retina, most of the cones have been lost and the remaining cones lack outer segments. If the remaining cones are required for entrainment then one must propose a mechanism that can compensate for massive photoreceptor loss and loss of outer segments (108).

A role for cones in photoentrainment has been explicitly examined in very recent experiments. Again transgenic mice were used, containing in this case an integrated fusion gene consisting of a portion of the human red cone opsin promoter (109) linked to an attenuated diphtheria toxin gene. Analysis of the retinae of these mice shows that the rod photoreceptors remain intact, UV cones are reduced, and green cones are eliminated (110). Despite the loss of green cones, the circadian system of these mice show saturating and subsaturating phase shifts in response to monochromatic green light (λ_{max} 515 nm) that are similar to congenic +/+ controls (Freedman, Soni and Foster, unpublished). These data suggest that there may be complete redundancy of the photoreceptor inputs to the clock. In the absence of the cones, rods mediate photoentrainment and with no rods, cones perform this task. Alternatively, the possibility that neither the rods nor cones are required for photoentrainment cannot be excluded. To test the possibility that there may be novel photoreceptors/photopigments within the mammalian eye, the rodless *rdta* mice and green coneless transgenic mice were bred and the progeny of these genetic crosses examined. Preliminary results show that green monochromatic light stimuli (λ_{max} 515 nm) are still capable of phase-shifting circadian behavior in these mice (Freedman, Soni and Foster, unpublished). If confirmed, these results provide strong evidence that the mammalian eye contains additional, not yet identified photoreceptors that may be specialized to mediate photoentrainment of the circadian system.

The possibility that the mammalian eye contains novel photopigments is made marginally less fantastic by the recent discovery of novel opsins within the eyes of other classes of vertebrates. A novel opsin gene has been isolated from the eyes of Atlantic salmon, and a comparative analysis of DNA from different species (by Southern blots) suggests that this opsin may be widely distributed in the fish and perhaps other vertebrate classes. The cDNA sequence predicts a protein that has the key features of an opsin but shows only 32–42% amino acid identity to the known opsin families. In addition, phylogenetic analysis suggests that this opsin is a member of an unrecognized opsin family that diverged from a common ancestor before all of the other known opsins (111). The discovery of this "vertebrate-ancient" (VA) opsin family may ultimately lead to an understanding of how vertebrate eyes indeed mediate photoentrainment.

Perhaps the most striking observation to emerge from the studies outlined above is that mice that lack classical visual responses are still capable of regulating their circadian rhythms by light with unattenuated sensitivities. However, these types of observation are not restricted to mice. Several studies have demonstrated that clock-driven rhythms can be regulated by light in a subset of humans that have their eyes but remain "blind" to visual stimuli (112). In addition, the blind mole rat (*Spalax ehrenbergi*) provides a further example of a mammal lacking classical visual responses but

that is still capable of photoentrainment (113–118). Results in retinally degenerate mice, mole rats and blind humans have led to the realization that two functionally distinct systems exist for processing light information in the mammalian eye (and perhaps the eye of other vertebrates): the "image-forming" photoreceptor system, which constructs a representation of the environment (classical vision) and the "non-image-forming" photoreceptor system, which detects changes in the overall quantity and quality of light at twilight. In view of the different sensory demands of image detection and the regulation of biological clocks it is not surprising that two systems for processing light information have evolved.

Although it is clear that the processing of light for image detection and twilight detection are different, we have not yet defined which features of twilight are utilized by the mammalian circadian system. Do mammals use the amount or spectral quality of light or the position of the sun for twilight detection, or a combination thereof? To gain an accurate measure of the phase of twilight, the circadian system needs to measure environment irradiance. Clear evidence for irradiance detection comes from an analysis of the morphology of the retino-hypothalamic tract (RHT). The retinal ganglion cells (RGC) projecting to the SCN (variously called type III in the rat and mouse or W-cells in the cat) are relatively scarce, are spread evenly across the retina and have extensive dendritic arbors (119,120). This would effectively reduce the spatial resolution of the projection but increase the sampling area. In addition, there is an absence of retinotopic order in RHT. The RGC project randomly to the retinorecipient areas of the SCN, which further blurs any image. These combined effects provide the SCN with irradiance information (121).

In addition to large changes in irradiance, there are very precise spectral changes associated with twilight. The freshwater algae *Chlamydomonas* (70) and the marine alga *Gonyaulax* (79) appear to utilize this spectral information to regulate their circadian clocks, but there is little evidence for this in the vertebrates. We do know that the circadian systems of the mouse (107) and the hamster (122) are sensitive to both green light and near-UV irradiation, but we do not know how the signals from these different spectral channels are utilized by the mammalian circadian system. Finally, the position of the sun could be utilized for twilight detection. We know that many animals can use the position of the sun for time-compensated sun-compass orientation (123), but whether this information is also used by mammals to entrain circadian systems remains a mystery. For this radiance detection task, topographic mapping, would be required to determine the position of the sun—perhaps in this way the "classical" visual system does contribute to photoentrainment.

CONCLUDING REMARKS

The mechanisms of the circadian system will only be understood comprehensively if we unravel the mechanisms of all of the parts of this system – the input, the oscillator and its outputs – especially because they might not be quite as separable as initially thought. Research of the molecular components of the rhythm-generating mechanism often assumes common features among the different circadian systems (*e.g.* negative transcriptional feedback loops in cyanobacteria, fungi, insects and mammals). However, all circadian systems are intimately associated with their photoreceptors, and, as we have outlined here, these appear to have evolved independently of the image-forming visual senses or those that are used for spatial orientation in the most divergent plant and animal species. It is reasonable to assume that the photosystems responsible for circadian entrainment are phylogenetically older than those for spatial discrimination. It may even be possible that photic inputs with multiple pigments for wavelength discrimination evolved originally as means of detecting changes in twilight, and only later became utilized for contrast detection in the image-forming visual systems.

Because of the close relationship between light input and circadian oscillator, it seems likely that photopigments and their transduction mechanisms have influenced the evolution of the "clock molecules." Different photopigments mediate entrainment in different groups of organisms: phytochromes and still unknown pigments in plants, opsin- and vitamin A-based photopigments in animals and probably flavines in the fungi. In view of these profound differences in the circadian light transduction pathways and their close relationship to the circadian system, it seems likely that this system has evolved in different species analogously but independently during evolution. Thus, analogous (and most likely not homologous) features will also be found for the clock molecules of bacteria, plants, fungi and animals and their transcriptional, translational, or posttranslational regulation (as they exist for many other functions as well). Only the clock molecules of closely related organisms (*e.g.* all vertebrates) are likely to be homologous.

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