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# The Art of Entrainment

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**Abstract** The circadian system actively synchronizes the temporal sequence of biological functions with the environment. The oscillatory behavior of the system ensures that entrainment is not passive or driven and therefore allows for great plasticity and adaptive potential. With the tools at hand, we now can concentrate on the most important circadian question: How is the complex task of entrainment achieved by anatomical, cellular, and molecular components? Understanding entrainment is equal to understanding the circadian system. The results of this basic research will help us to understand temporal ecology and will allow us to improve conditions for humans in industrialized societies.

**Key words** circadian, oscillator theory, clock genes, clock proteins, feedback loops, photoperiodism, PRC

## WHAT'S THE PROBLEM?

The ability to oscillate with a self-sustained amplitude in constant conditions is regarded as the circadian clock's central quality, yet in nature, the opportunity for free-running rhythmicity (which occurs in the absence of environmental cues) is surely rare. Rather, the entrained oscillation prevails. That circadian periods in constant conditions can substantially deviate from 24 h led to the often-repeated reasoning that entrainment is necessary *because* clocks have to be corrected. This viewpoint may be misleading: The circadian clock is not entrained because its free-running period deviates from 24 h, but it is able to free run because of how it evolved to work optimally when entrained. This is supported by the fact that there is no *exact* circadian period because it depends on the nature of the constant conditions (e.g., constant light vs. constant darkness; Aschoff, 1951). Thus, the correct statement should be the following: The circadian clock has evolved to fine tune biological functions to specific times within the day or night, *and*, when put into constant conditions, it free runs close to 24 h! To fully

appreciate the function of the circadian system, we have to understand how clocks entrain to the environment.

The issue of entrainment was very much at the heart of early circadian research (see many contributions to the proceedings of the Cold Spring Harbor Symposium, "Biological Clocks," 1960), whereas only certain aspects of entrainment have been part of the molecular genetic approach to understanding the clock. These are mainly related to entrainment by light: (1) light regulation of "clock molecules" (e.g., Crosthwaite et al., 1995; Hunter-Ensor et al., 1996) or light induction of immediate early genes (Rusak et al., 1990; Meijer and Schwartz, 2003 [this issue]), (2) the close association of the molecular clock mechanism with light input pathways (Crosthwaite et al., 1997), and (3) specialized photoreceptors involved in light entrainment (Berson et al., 2002; Freedman et al., 1999). Yet how light entrains the circadian clock at the molecular level remains an open question.

So what is entrainment and how does it differ from simple synchronization? Any system could be synchronized to daily environmental changes simply by

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passively responding to changes in light and/or temperature (e.g., by using a timer that is triggered each dawn). In contrast, the process of entrainment refers to a system that is itself an oscillator (hence, the potential to free run) and that responds discriminately to those external stimuli that specifically can reset the circadian clock (*Zeitgeber*, "time giver" in German). The clock's responses are formally predictable (see below) and are quite distinct from passive ones. For example, oscillators may take several cycles until they reach synchrony with the zeitgeber, and once they do, they establish a predictable phase relationship to it. The term *phase* relates to times within an oscillation. The phase of a cycle can be defined by any reference point of an observable rhythm. Within zeitgeber cycles, dawn or dusk are frequently used as external reference points, while the onset of activity or the maximum of a rhythmic protein could serve as internal reference points. The *phase of entrainment* relates internal and external phase and thus defines the relative timing of a given circadian event (e.g., activity onset) within the external 24-h day. The phase of entrainment is not fixed but shows plasticity, which depends on several parameters: the period of the endogenous oscillator ( $\tau$ , the amount of time it takes for a full cycle in constant conditions), the period of the zeitgeber cycle ( $T$ ), its proportion of light and darkness (photoperiod), as well as the zeitgeber strength or amplitude. Due to the predictability of responses by circadian clocks to zeitgeber signals, the pioneers of circadian research compared circadian clocks to physical oscillators (Aschoff, 1959) and used the underlying mathematics to explain results of entrainment and resetting experiments.

Although most organisms synchronize to their environment by entrainment, not all of their rhythmic features are synchronized via clock control: Direct effects of light (or other stimuli) can mask the expression of the clock. Many animals become active or inactive just because lights go on or off. Their apparent phase of entrainment then coincides with one of the zeitgeber transitions. To distinguish between masking and clock control, entrained organisms are commonly released to constant conditions, and the onset of free-running activity is extrapolated back to the last day of entrainment. When it does not coincide with the apparent phase of entrainment, circadian control was masked by direct light effects. Although masking features aspects of oscillatory behavior (Aschoff and Goetz, 1988), it appears to involve mechanisms differ-

ent from those that control the circadian system (Mrosovsky, 2001).

The basic, formal rules of entrainment are as important for molecular circadian research as they were for the initial understanding of the system. Genetic and molecular components of the circadian system have to be investigated for their role in entrainment. Which components receive the zeitgeber stimulus first (e.g., are light induced or destroyed by light) and how do they shift the phase of other clock components? Are there components that contribute more to advancing the oscillator and others more to delays? Do some respond preferentially to dawn and others to dusk? And finally, entrainment and masking have to be distinguished on the molecular level, as they are on the systemic level. A synopsis of the formal properties is, therefore, summarized in this introductory article of the special issue on entrainment.

### LIKE MECHANICAL OSCILLATORS

Any oscillator, including a circadian system, can, to some extent, be compared to either a pendulum or a swing. Consider a swing that swings 12 h from left to right, for example, from dawn to dusk (Fig. 1A) and 12 h back (from dusk to dawn). If the swing is pushed (for a negligibly short time), its speed transiently changes, and depending on when the push is given, different phase shifts result. The response of any oscillator to a perturbation depends on phase. These systematic responses can be graphed as a phase response curve (PRC) (Fig. 1B).

A PRC is a graph depicting phase shifts that move a system forward, backward, or not at all in response to a stimulus. Pushing the swing when it is moving fast, in the middle position (Fig. 1A), mainly changes the amplitude of its oscillation and has little effect on phase. Pushes given when the swing stops at the extreme positions will cause phase advances when directed toward the center (by convention positive values) and phase delays when directed away from the center (negative values). Pushes given at phase 6, when the swing has little speed, advance its phase (they are directed toward the center). When the swing picks up speed, the advancing effects decrease until they have no effect around phase 12. This so-called dead zone of the PRC exists only at phase 12 in a simple mechanical oscillator but can be much larger in PRCs of circadian oscillators (compare with Figs. 2A

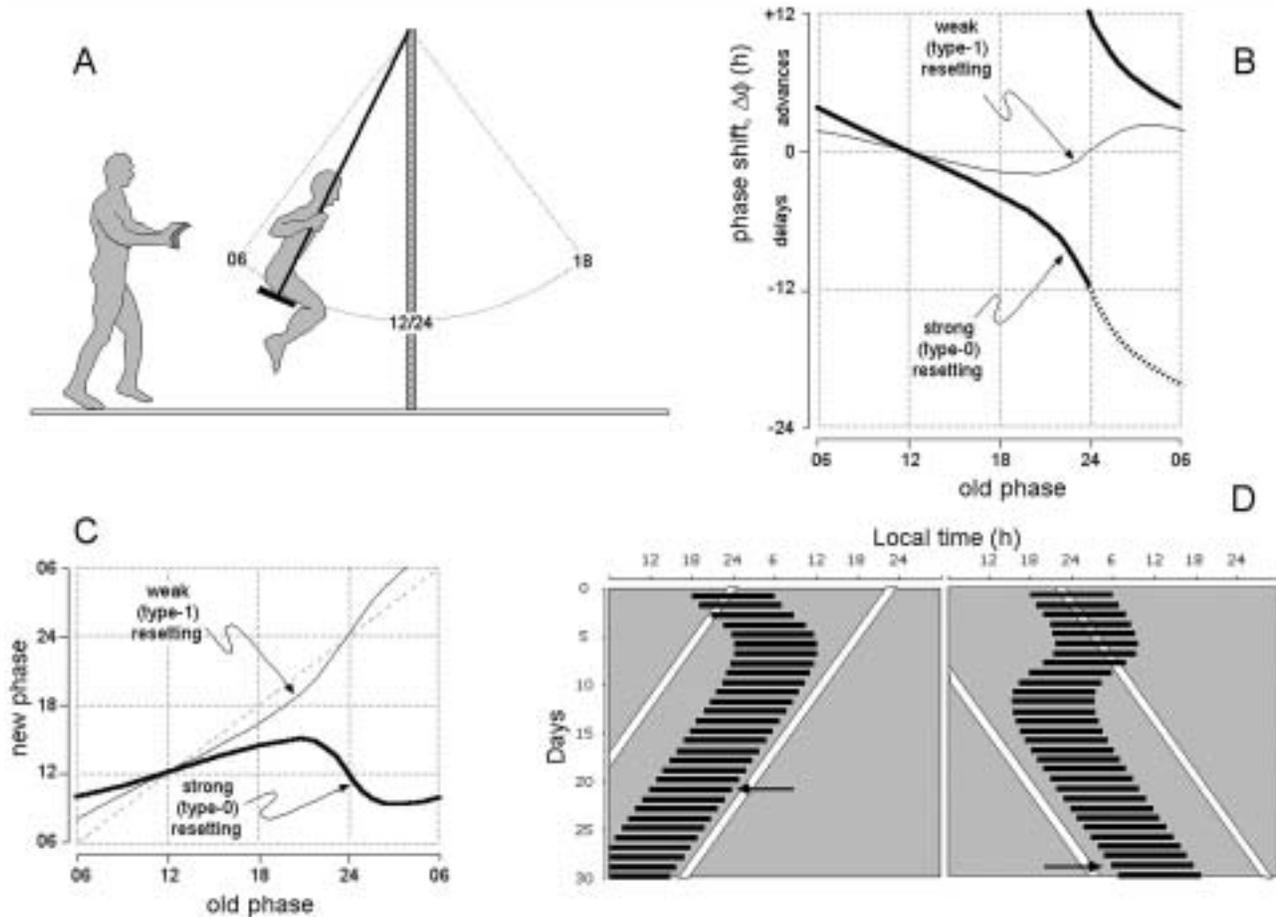


Figure 1. Phase response curves (PRCs) and principles of entrainment explained with the help of a swing oscillator. (A) Perturbations can be introduced by pushing the swing in one direction with a given force. (B) The phase shifts resulting from these perturbations are a function of the oscillator's phase and can be summarized in a PRC. When the pushes are strong enough, so they even can reverse the swing (e.g., with 1.5 times the swing's maximum velocity), the PRC reaches maximum phase shifts, that is, half a cycle, when hit at phase 24 (thick and stippled line). Weaker pushes (e.g., with 50% maximal velocity) result in a different PRC (thin line) (C) Alternatively, the perturbations can be drawn as a phase transition curve (PTC), where the new phase, resumed by the swing after the perturbation, is graphed as a function of the old phase at which it received the push. Strong resetting results in a curve with an average slope of 0 (type-0 resetting), while weaker pushes lead to new phases that lie, on average, around a line with a slope of 1 (type-1 resetting). (D) Entrainment of a swing with a 24-h period by repetitive, weak perturbations (white area) applied every 23 h (left panel) or 25 h (right panel). In these phase plots, the back swing of the pendulum is drawn as bars. The days on which stable entrainment is reached are indicated by arrows.

and B). When the swing decelerates again, a push will give it extra energy, increasing its amplitude and causing a delay. When the swing has turned (at phase 18), pushes will delay its phase even more.

The PRC for perturbations that are strong enough to reverse the swing's direction when traveling at full speed toward the pusher (thick line in Fig. 1B) is different from the PRC for weaker pushes (thin line). The largest differences between strong and weak pushes occur when the swing travels toward the pusher. For weak pushes, the delaying effects decrease again with increasing speed until they become ineffective at phase 24. After that, weaker pushes advance because

they decrease the swing's amplitude and make it turn sooner (at phase 06). If the push has just the right strength to stop the swing exactly at phase 0/24, a harmonic oscillator will stop dead (at its "singularity"; Winfree, 1970). A push harder than the singular force at phase 0/24 inverts the direction of the swing and will, therefore, shift its phase over half a cycle. In this case, a discrimination between advances and delays is not possible; the PRC for strong pulses can therefore also be drawn as delays only (see stippled continuation in Fig. 1B).

PRCs for mechanical oscillators as well as for circadian systems display phase shifts as a function of

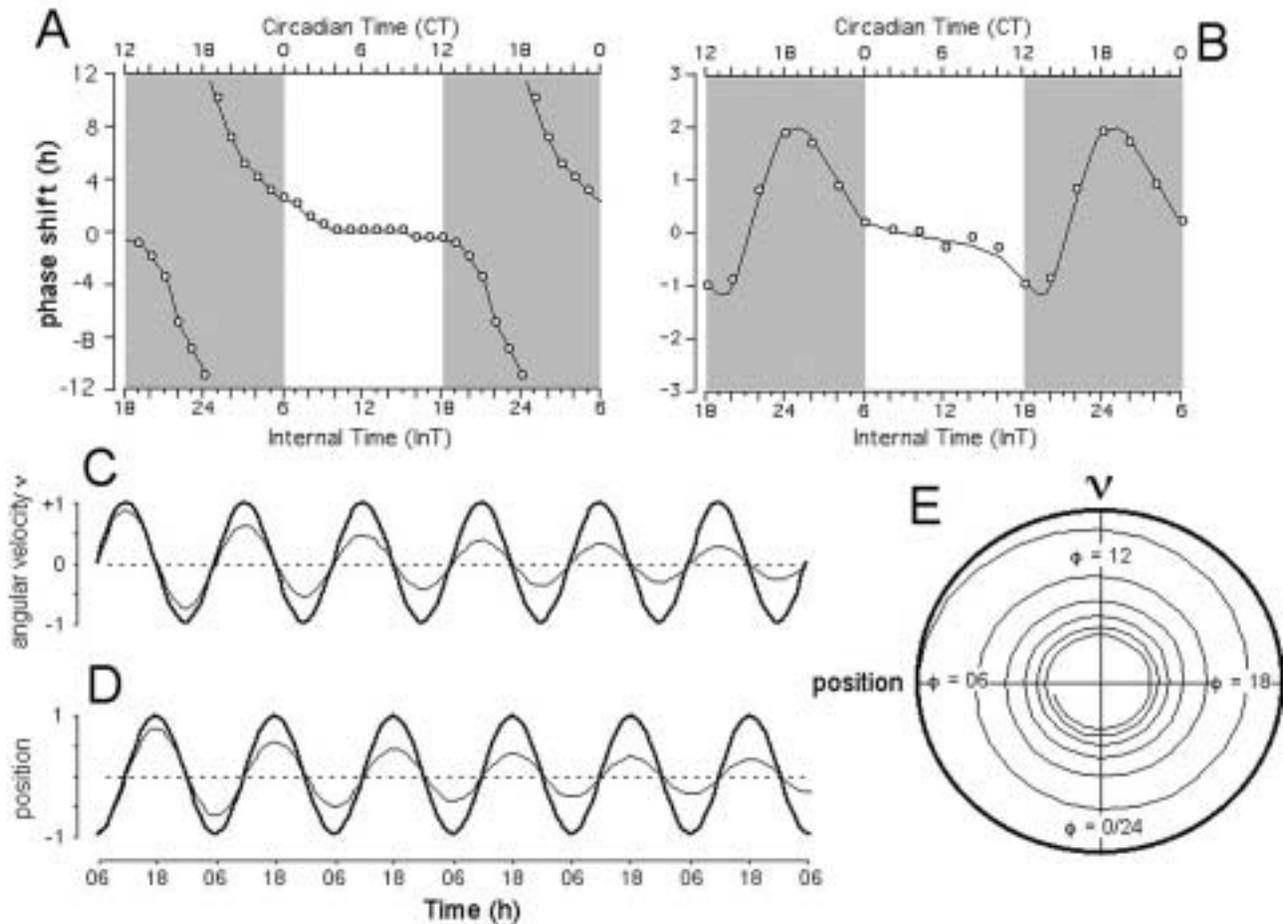


Figure 2. Phase response curves (PRCs), phase planes, and limit cycles. (A) Type-0 PRC of the pupal eclosion rhythm of *Drosophila pseudoobscura* (from Pittendrigh and Minis, 1964). (B) Type-1 PRC of the hamster activity rhythm (Daan and Pittendrigh, 1976a). Both PRCs (A and B) are plotted as a function of circadian time (CT) (top abscissa) and internal time (InT) (bottom abscissa). Unlike a swing, circadian oscillators do not damp (compare thin and thick lines in C-E). When the velocity,  $v$ , of the oscillator (C) is drawn as a function of its position (D), it is represented in a phase plane (E). Self-sustained oscillators form a limit cycle to which the system returns (thick line in E), while harmonic oscillators are damped and gradually spiral inward with decreasing velocity and amplitude. Note that while the position of a harmonic oscillator changes with decreasing amplitude (D; it does not swing as far), the phase (e.g., when it reaches a maximum) does not.

phase (Fig. 1B). Phase shifts can also be drawn as a phase transition curve (PTC), where the new phase that the oscillator adopts after the perturbation is drawn as a function of the old phase at which it was perturbed (Fig. 1C). PTCs for strong and for weak resetting have distinct slopes. In the former case, the average slope is 0 (thick curve), while in the latter case, phase shifts undulate around a diagonal line (slope = 1). Based on the slopes of their PTCs, strong resetting has also been called "type-0" and weak resetting "type-1" (Winfree, 1970).

While PRCs show the effects of a single perturbation, entrainment results from regularly repeating perturbations. Entrainment of oscillators can be accu-

rately predicted on the basis of PRCs (Daan and Pittendrigh, 1976b; Pittendrigh and Minis, 1964). This is true for entrainment of circadian systems by zeitgebers (e.g., a light:dark cycle) and also holds for mutual entrainment ("coupling") of oscillators such as 2 SCN neurons. That oscillators establish different phases of entrainment depending on their period ( $\tau$ ) and that of the zeitgeber cycle ( $T$ ) is predicted from the PRC. In Figure 1D, the repetitive perturbations are drawn as white areas (exemplifying a daily light pulse), and each second half of the oscillation is drawn as a bar (e.g., representing activity of a nocturnal animal). The oscillator has an endogenous period of 24 h ( $\tau = 24$ ), and the period of the zeitgeber is either shorter

or longer ( $T = 23$ , left panel in Fig. 1D, or  $T = 25$ , right panel in Fig. 1D). This is unnatural, but it makes the math simpler. Stable entrainment is reached when the phase shift ( $\Delta\phi$ ) corrects for the difference between  $\tau$  and  $T$ ,  $\Delta\phi = \tau - T = 24 - 23 = +1$  or  $\Delta\phi = \tau - T = 24 - 25 = -1$  (arrows in Fig. 1D). The examples in Figure 1D are based on the type-1 PRC (thin line) shown in Figure 1B, with the first pulse arbitrarily given at phase 23.

When the perturbations recur every 23 h ( $\tau > T$ ; left panel in Fig. 1D), they gradually “walk” to the left, through the delay portion and the flat zone of the PRC into its advance portion until they reach (after 21 cycles) the phase of the PRC, where  $\Delta\phi = +1$  h (shortly after the end of activity). Only in this configuration will pulses recurring every 23 h hit the system at exactly the same (stable) phase. When the perturbations reoccur every 25 h ( $\tau < T$ ; right panel in Fig. 1D), they gradually “walk” to the right through the advance portion and the flat zone of the PRC until they reach  $\Delta\phi = -1$  h on day 29 (shortly before the onset of activity). Note the huge difference in the phase of entrainment that is derived from the same PRC but using a zeitgeber with a different period.

In the given examples,  $T$  is varied and  $\tau$  remains constant, while in real life,  $T$  remains constant and  $\tau$  can vary. The phase of entrainment is similarly predictable from the  $\tau/T$  relationship: The shorter  $\tau$ , the earlier its phase of entrainment (e.g., the time of the body’s daily temperature minimum or the melatonin peak relative to dawn). This has been demonstrated for dozens of organisms, including humans: Those who like to go to sleep and get up early (morning types) tend to have a shorter free-running period than those who prefer to sleep later (evening types; Duffy et al., 2001).

The PRCs measured for circadian systems of all phyla (see examples in Fig. 2) closely resemble those derived for the swing example (Fig. 1B). Both type-1 and type-0 PRCs can be found depending on the organism and the strength of the pulse. Because free-running periods may deviate from 24 h, the progression of the endogenous circadian rhythm must be described with its own time base. The half of the endogenous cycle (in constant conditions) that coincides with daytime under entrained conditions is called “subjective day,” and the other half is called “subjective night.” Just as the positions of the swing (Fig. 1A) have been labeled phases 0 to 24, the phases of the endogenous circadian cycle (regardless of its endogenous period,  $\tau$ ) are designated internal time

(InT) 0 to 24 (Daan et al., 2002). Similarly, a zeitgeber cycle (regardless of its length, which can be varied in experiments) is divided into 24 h of external time (ExT), starting from midnight. In symmetrical LD cycles only (e.g., LD 12:12), ExT equals ZT + 6 and InT is CT + 6 (compare lower and upper abscissa in Fig. 2 A,B). ZT and CT are traditional and widely used conventions, both starting to count with the onset of light. The new convention was proposed because ExT 0 always coincides with the middle of darkness and ExT 12 with the middle of light, irrespective of the length of light (photoperiod) or darkness (scotoperiod).

### NOT LIKE MECHANICAL OSCILLATORS

In spite of the similarities between the PRCs of simple oscillators and the PRCs of circadian clocks, obviously there are profound differences. While few variables contribute to the behavior of a simple oscillator (e.g., the length of the pendulum), the circadian system is highly complex, involving many components at the anatomical and at the molecular level. Furthermore, any mechanical oscillator will damp out as it loses energy due to friction. Damping affects both velocity ( $v$ , thin line in Fig. 2C) and amplitude (thin line in Fig. 2D) but does *not* affect period. In mechanical clocks, damping is prevented by an escapement that transfers energy to the pendulum (e.g., from a weight), maintaining velocity and amplitude (thick lines in Fig. 2 C,D). When the velocity of the oscillator is drawn as a function of position in a phase plane (Fig. 2E), self-sustained oscillations trace stable limit cycles (thick line) while damped oscillations gradually spiral inward (thin line). Thus, self-sustained biological clocks, such as in the SCN, are stable limit cycle oscillators that spontaneously revert to a constant amplitude when perturbed or even after being stopped. This is in contrast to damped oscillators and apparently also to the damped circadian rhythms found in peripheral organs (Yamazaki et al., 2000).

Perturbations of a limit cycle oscillator transiently change its velocity and/or position, but the system quickly returns (is attracted) to its inherent limit cycle, progressing through time (see the numbers indicating different phases in Fig. 2E). During transients, the system will respond according to a different PRC, but once back on its limit cycle, the PRC is identical to the one it had before the perturbation. Systems that always respond with the same PRC are called “phase-

only systems." Thus, the stronger the attraction to the limit cycle, the faster the system returns and the more it behaves like a phase-only system. Predicting circadian entrainment on the basis of PRCs alone (as in Fig. 1D) implies such a phase-only system. Using 2 consecutive light pulses (double pulse experiments), the assumption of rapid return has been found valid for the *Drosophila pseudoobscura* eclosion rhythm (Chandrashekar, 1967). For short light pulses recurring once every 24 h, one can readily and precisely predict details of entrainment on the basis of a phase-only model (e.g., Pittendrigh, 1981).

However, circadian systems do not always behave like phase-only systems. Although Winfree (1973) confirmed the observations for *D. pseudoobscura* eclosion, he observed that the return is much slower after pushing the system further away from the limit cycle either by prolonged continuous illumination (LL) or by a brief light pulse tuned to push the system onto the point of singularity. Phase-only models are insufficient to predict circadian entrainment for long perturbations (e.g., 12 h of light) because different light levels cause different velocities of the circadian oscillator (parametric effects; Daan and Pittendrigh, 1976b). While the phase-only model is based on single short pulses and considers only the nonparametric influences, entrainment in the real world must be based on both nonparametric and parametric effects (Aschoff, 1963) or even primarily on parametric effects (e.g., by tracking changes of light intensity over the course of the day; Hut et al., 1999).

The phase-only model also fails to predict entrainment by frequent perturbations. If, for example, the swing was pushed every hour at phase 19 back to phase 18 ( $\Delta\phi = -1$ ; see Fig. 1A), it would get stuck between these 2 phases. However, in reality, circadian systems continue to oscillate close to their endogenous period (e.g., Aschoff, 1999; Eriksson and Veen, 1980). Entrainment has to correct for the difference  $\tau - T$  on a daily basis. When a zeitgeber cycle is too short or too long, this difference may be larger than the maximum delays or advances of the PRC, so that a circadian clock cannot establish a stable phase of entrainment. Outside of their range of entrainment, clocks tend to freerun with regular interactions with the zeitgeber (relative coordination; Holst, 1939). Even zeitgeber cycles with  $T = \tau/2$  are outside the circadian range of entrainment, so that circadian clocks typically demultiply the frequency. In this case, 2 consecutive zeitgeber perturbations add up to correct the endogenous period to  $2 \times T$ .

Phase-only models also do not predict circadian behavior under extreme photoperiods. When dawn and dusk are presented as only 2 light pulses ("skeleton" photoperiods), activity ( $\alpha$ ) occurs only within 1 of the alternative intervals. When this interval is systematically reduced, the subjective night of woodmice (*Peromyscus*) is compressed more than is predicted from the PRC (Pittendrigh and Daan, 1976b), again calling for more complex paradigms. For example, onset and end of activity may be controlled by separate oscillators within the circadian system (evening [E] and morning [M]; Pittendrigh and Daan, 1976c).

More generally, predicting entrainment from the PRC (phase-only model) assumes that both the PRC and  $\tau$  are constant, intrinsic properties of the system. This is probably rarely true since both the PRC and  $\tau$  are affected by entrainment, which can be observed when the organism is released to constant conditions (after effects). For instance, when mice are released from long or short zeitgeber cycles to constant darkness (DD),  $\tau$  is changed for up to 100 days (Pittendrigh and Daan, 1976a). Inversely, constant conditions can have major effects on the PRC, for example, in hamsters kept in DD for a long time (Pittendrigh, 1981). In summary, the light (and/or dark) history of an organism has pronounced effects on the detailed patterns of entrainment and helps to tune the system to its environment (Beersma et al., 1999).

#### ENTRAINMENT: SYNCHRONIZATION WITH MANY DEGREES OF FREEDOM

The active process of entrainment, in contrast to a passive response, creates flexibility in temporal organization and thus has adaptive potential. A change in the phase of entrainment could be achieved, for example, by simply speeding up or slowing down the progression of the endogenous rhythm because the phase of entrainment depends on  $\tau/T$  (see above). Songbirds considerably shift the onset of activity forward each spring, related to their dawn chorus in reproduction (important for territorial advertisement and mate attraction early in the morning). Whether this shift is accomplished by changes in the circadian pacemaker or in the outputs controlling behavior remains to be established (Daan and Aschoff, 1975).

The phase of entrainment is not only affected by  $\tau$  (see Fig. 1D) but also by the strength of the zeitgeber, for example, by the amplitude in day-night light intensity differences. The effect of zeitgeber strength

on phase of entrainment again depends on the individual's free-running period. With decreasing zeitgeber strength, the clock will move forward to an earlier time if  $\tau < 24$ . For  $\tau > 24$ , typical for most humans, the clock will, conversely, move sleep and activity to a later phase. Thus, the distribution of chronotypes becomes broader with decreasing zeitgeber strength. In some humans, the phase of entrainment affects normal integration into everyday life (Advanced or Delayed Sleep Phase Syndrome). Individuals suffering from these syndromes regularly wake up as early as 4 AM or, respectively, cannot fall asleep until 3 AM (e.g., Ebisawa et al., 2001; Toh et al., 2001). Such extreme entrained phases may be exacerbated by decreasing zeitgeber strength in industrialized societies (Roenneberg et al., 2003).

Thus, the phase of entrainment can vary for different reasons, each related to different parts of the circadian system: (1) the zeitgeber signals may be received or transduced with different efficiency, for example, due to genetic differences in the receptor or the transduction cascade; (2) clocks may (genetically) have or (adaptively) adopt different free-running periods; or (3) outputs may be differently coupled to the circadian clock.

### FROM TIME GIVERS TO CLOCK MOLECULES

Light probably represents most reliably the progression of day and night in most environments since the timing of many other rhythmic cues depends on sunlight (temperature, most weather and climatic factors, or food sources). Yet zeitgebers other than light play an important role in entraining circadian clocks and can interact with the zeitgeber light. These interactions add another level of complexity to the art of entrainment. Light and temperature cycles, for example, mutually interact in the entrainment of *Neurospora* (Liu et al., 1998; Roenneberg and Merrow, 2001).

Even environmental factors that are not zeitgebers in a strict sense because they do not oscillate in nature may contribute to entrainment. This is the case when exposure and/or uptake of environmental factors are under circadian control. The unicell, *Gonyaulax polyedra*, is exposed to higher nitrate concentrations only during the night, when the cells sink to lower depths during their daily vertical migration. Under constant conditions, rhythmic nitrate exposure entrains the *Gonyaulax* clock, and both light and

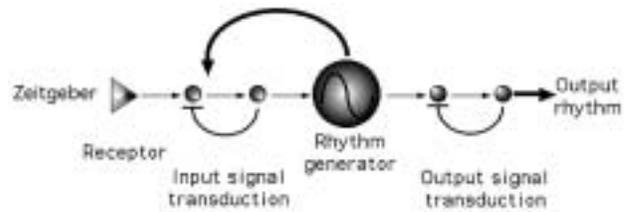


Figure 3. The circadian pathway. The circadian system can be depicted as a pathway with the mechanism that generates the circadian rhythmicity at its center, receiving entraining signals via a specific receptor and transduction pathway and controlling output rhythms by sending signals down another transduction pathway. Besides the molecular feedback loops that form the basis of generating a circa 24-h rhythmicity, other feedback loops may be part of the transduction pathways or provide feedback from the rhythm generator to the input pathways.

nitrate mutually interact (for references, see Roenneberg et al., 1998). Modification of entrainment with light has also been shown for the nonphotic effects of activity on entrainment (Mrosovsky, 1991) or for entrainment by periodic food intake (Stephan, 1986).

Light is most effective in circadian resetting during the subjective night. This can be predicted from the PRC since the most sensitive portion of the PRC, that is, the portion yielding the largest phase shifts, always shifts the oscillator away from the time of day when the signal is most prominent. Because this is a pacemaker property, it concerns both diurnal and nocturnal organisms and holds for many different stimuli (see the PRC atlas, Johnson, 1990): dark or temperature-down pulses are more effective during the subjective day, while temperature-up pulses elicit larger phase shifts during the subjective night. It even holds for signals that become zeitgebers only by exposure; nitrate is more effective during the subjective day for *Gonyaulax* when the cells normally are found in the nitrate-poor upper layers of the ocean.

Light is generally less effective in phase shifting during the subjective day than would be predicted for a simple oscillator, as manifest in the larger dead zone of circadian PRCs (compare Fig. 1B with Figs. 2 A,B). This may be due to the many feedback loops within the circadian pathway beyond those that generate circadian rhythmicity (Fig. 3). For instance, circadian systems often control their own inputs with the consequence that signals are received and transduced with different sensitivity or efficacy at different circadian phases. In plants, for example, expression of light receptors is controlled by the clock (see Millar, 2003

[this issue]), and in *Neurospora*, clock and light input pathway components are inseparable (see Liu, 2003 [this issue]). Thus, rhythm generator and input pathways are not easily distinguishable in such a network: While the inputs change the qualities of the clock, the clock changes the properties of the input. In fact, the observed phenotypes of clock mutants (arrhythmicity, altered period length, or loss of temperature compensation) can also be modeled when the clock components function as part of an input pathway under circadian control (Roenneberg and Merrow, 1998).

Unlike in most other animals, light reaches the circadian system in mammals exclusively through the eyes (Nelson and Zucker, 1981). Yet light entrainment persists in mice without rods and cones (Berson et al., 2002; Freedman et al., 1999). The race to identify the responsible light receptor(s) has reached the final laps, indicating melanopsin as one likely circadian photopigment (see Rollag et al., 2003 [this issue]). However, light-dependent entrainment and pupillary restriction still persist, although reduced, in melanopsin knock-out mice. Only combinations of genetic deficiencies of the known mammalian light receptors will answer the question of how they interact and whether additional, nonvisual photopigments still remain to be discovered. In contrast to recent statements (News and Editorial Staffs of *Science*, 2002), the issue of how light is received and reaches the molecular clocks in the mammalian SCN cannot be "put to rest."

Conceptualizing the circadian system as a pathway (Fig. 3) works on several levels. At the systemic level in mammals, the receptor resides in the retina and the pacemaker in the SCN, while receptor(s) and rhythms generator(s) are inherent to the cell for cellular clocks. For the system, the zeitgeber is exogenous (e.g., light), while the entraining signals for cellular clocks are endogenous (transmitters, hormones, etc.). In the case of the liver clock, both signals from the SCN and cues from feeding and metabolism contribute to entrainment (see Schibler et al., 2003 [this issue]). Hence, the nature of entrainment is distinct for different tissues. This could serve an adaptive function, for adjusting to different timing of food sources, to changing photoperiod and seasons.

In mice, light appears to affect the circadian molecular network via the induction of 3 genes (*Per1*, *Per2*, and *Dec1*) in the SCN (Honma et al., 2002; Shigeyoshi et al., 1997; Zylka et al., 1998). Light received via retinal photoreceptors is transmitted to the SCN via 2 pathways: directly via the retino-hypothalamic tract using

glutamate and PACAP as principal neurotransmitters and indirectly via the IGL and the midbrain using GABA, neuro-peptide Y, and serotonin as transmitters (for references, see Reppert and Weaver, 2001). The intracellular transduction pathways appear to involve Ca<sup>2+</sup>-mediated phosphorylation of CREB, which binds to cAMP-responsive elements of the promoters of *Per1* and *Per2*. All 3 light-regulated clock genes are induced (with very distinct kinetics) at specific circadian times: *Dec1* throughout the subjective night, *Per1* both at the beginning and the end of the subjective night, and *Per2* only in the early subjective night. These differences suggest that the 3 light-inducible genes of the mammalian circadian system perform different functions and may even receive zeitgeber information via different transduction pathways, possibly involving different light receptors. In addition, they may be spatially differently distributed over subareas of the SCN (LeSauter and Silver, 1999). An important open question is how individual input genes fine-tune entrainment; they may be responsible for different light responses (e.g., delays and advances), or the measurement of day length, by separately responding to dawn and dusk (Daan et al., 2001).

#### UNDERSTANDING ENTRAINMENT = UNDERSTANDING THE CLOCK

It is remarkable that both the clock mechanism and the light input pathway to the circadian clock appear difficult to destroy. While single mutations or knock-outs of clock genes appeared to abolish self-sustained rhythmicity, further experiments have shown that many circadian qualities remain intact (e.g., Merrow et al., 1999; Yoshii et al., 2002) or reappear when tested in different conditions (Spoelstra et al., 2002; Steinlechner et al., 2002) or when additional clock mutations are introduced (Oster et al., 2002). Similarly, inactivation of light input components does not lead to complete "blindness" of the clock (see many of the contributions in this issue). The circadian pathway shown in Figure 3 is more complex than the original input-output scheme but is still insufficient to explain all the experimental results. The clock consists of a network of feedbacks (Roenneberg and Merrow, 2003) connected to multiple receptors and input pathways, and it may be helpful to create a taxonomy of possibilities concerning both the anatomical and the molecular level (Fig. 4, the complexity of the input increases from

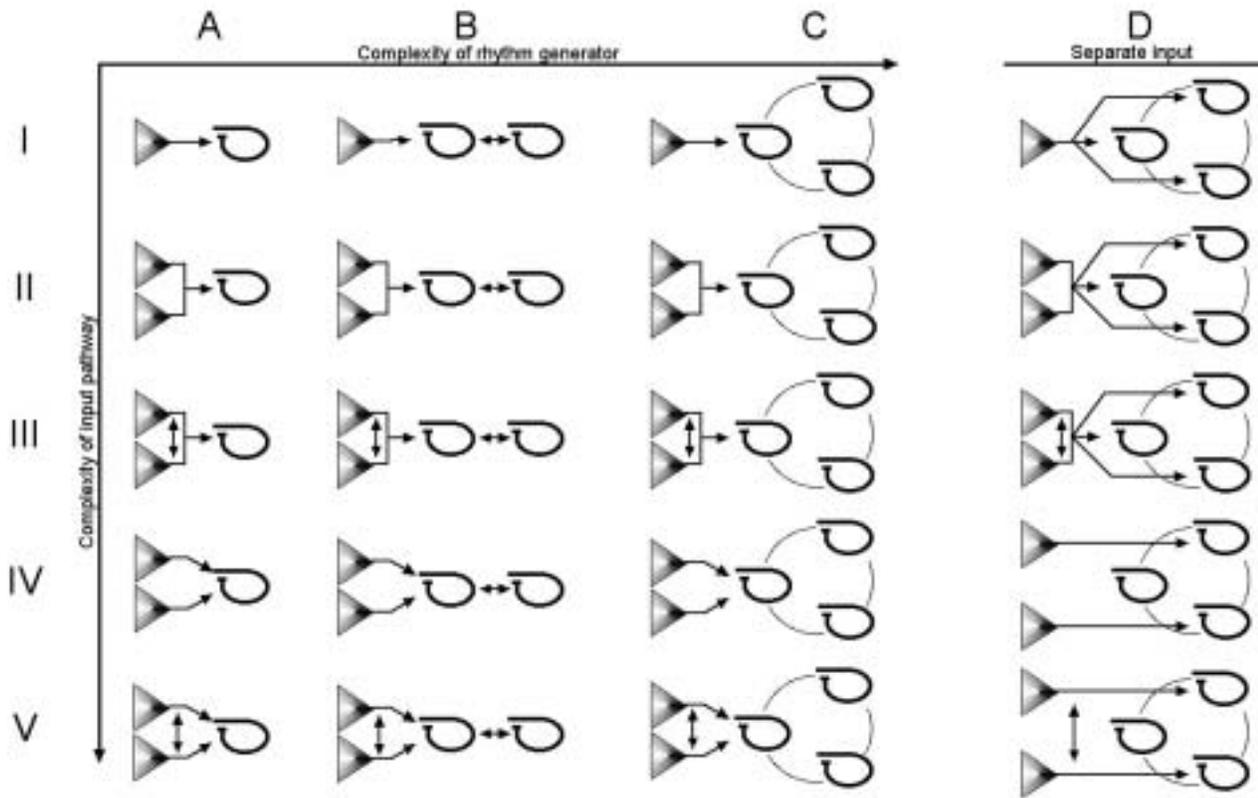


Figure 4. Possibilities of how the input is connected to the clock. The matrix formally shows how different numbers of receptors (and input pathways) can be connected to a clock mechanism containing one to several feedback loops. The number of inputs increases from top to bottom (I-V), the number of feedbacks from left to right (A-C). In column D, the complexity is increased by light inputs connecting separately to different feedbacks.

top to bottom and that of clock mechanisms from left to right). The 2 feedbacks in column B could represent a retinal clock and the SCN, SCN core and shell, or the FRQ- and the FRQ-less oscillator in *Neurospora* (Iwasaki and Dunlap, 2000).

Diagram A-I in Figure 4 represents the simplest possibility: a single input connected to a single feedback. Although row II represents systems with multiple receptors, the clock receives a single (fused) signal, for example, inducing a clock gene (the receptors act as an antenna collecting photons over a wider spectral range). In row III, the signals received via multiple receptors interact before they fuse and could, therefore, detect spectral qualities (rudimentary color reception). In rows IV and V, the multiple inputs stay separate (e.g., interacting with different light-responsive elements of a clock gene promoter). Regardless of the level of complexity, input signals affect only one feedback of the molecular network in columns A to C, while different feedbacks are directly affected in column D.

The complexity of circadian systems is likely to have increased over the course of evolution. Except for cyanobacteria (Schmitz et al., 2000), all circadian model systems appear to be comprised of more than one (coupled) feedback loop, excluding them from column A, and appear to receive light via more than one input, excluding them from row I. This is true for *Gonyaulax* (Roenneberg and Hastings, 1988; Roenneberg and Morse, 1993), higher plants, *Neurospora* (Dragovic et al., 2002), insects, and mammals.

A formal analysis of these possibilities (Fig. 4) provides a framework for experimental results and facilitates their interpretation. In mammals, for example, at least 3 separate molecular feedbacks (Per1, Per2, and Dec1; for a description of circadian networks of feedback loops, see Roenneberg and Merrow, 2003) are regulated by light, possibly via different inputs (possibilities D-II to D-V). Using the taxonomy, 2 questions have to be answered: (1) Do the inputs interact before they reach the clock components? and (2) Are the

genes induced via separate input pathways? Experiments investigating the effects of single and combined receptor/input mutants on the response of the entire system would, for example, address question 1, and those investigating the induction and entrainment kinetics of the individual genes address question 2. Existing results indicate that (1) image-forming and non-image-forming light inputs interact (Russell Foster, personal communication March 3, 2003), (2) different clock genes are entrained with different transients (Reddy et al., 2002), and (3) entrainment of the *Per1* rhythm is instantaneous while activity rhythms show transients for many cycles (Yamazaki et al., 2000). Thus, an initial analysis would place the mammalian system into category D-V.

But how can the modern tools of molecular biology and genetics be used in conjunction with the formalisms described here to decipher the mechanisms involved in entrainment? Entrainment (in contrast to passive synchronization) relies on the existence of an oscillator with a period somewhere close to the zeitgeber cycle. Many of the molecular features, necessary to generate the circadian oscillation, have been described based on mutants. To understand the role of different circadian components in entrainment, we must determine how these mutants affect different characteristics of entrainment. Many clock mutants, for example, have different free-running periods and should, therefore, show different phases of entrainment. Although in some cases, different phases are suggested by available data, too few systematic experiments have been performed on mutants in clock and/or input components. In this context, it is important to distinguish between "masking" and clock control of the locomotor activity. This distinction is also relevant at the molecular level: Within a molecular network, some components may respond acutely to light (i.e., appear to be driven) despite the fact that they play an important role in maintaining a self-sustained rhythm in DD. The mammalian *Per1* gene, for example, is strongly light induced, so that a luciferase gene under control of the *Per1* promoter may report acute light responses rather than entrainment of the entire network (Yamazaki et al., 2000). Interactions between molecular clock components have often been concluded from their behavior in DD (e.g., the lag between RNA and protein). Many of these conclusions (for both wild type and mutants) have to be reexamined in time series collected under different entraining conditions, using different T-cycles and/or

different photoperiods (Suri et al., 2000). Interpretations based on simple release experiment from darkness to light or from light to darkness are only marginally useful in understanding entrainment on the molecular level (Collett et al., 2002). In addition, special emphasis should be given to the role of proteins (Nuesslein-Hildesheim et al., 2000) because neither entrainment nor the mechanisms underlying photoperiodism can be explained based on RNA profiles alone (Yanovsky and Kay, 2002). Another important focus should examine the behavior of different clock components relative to each other, for example, by using protocols that elicit typical circadian behavior under extreme entraining conditions. If, for example, a circadian output shows frequency demultiplication in an LD 6:6 cycle (entraining to  $2 \times T = 24$ ), some components may follow the light cycle, others the output rhythm. When different phases of entrainment are achieved by different zeitgeber strengths, the response of some components may directly reflect, for example, different light intensities while others will reflect the changes in phase. Which of the clock components progressively change their kinetics and relationship to other components in prolonged DD, so that they could be used to explain the changes of the PRC under these conditions? Which components contribute to the "temporal memory" of the system that becomes evident in after effects (Brandstätter et al., 2001)?

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