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Circadian systems: different levels of complexity

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After approximately 50 years of circadian research, especially in selected circadian model systems (*Drosophila*, *Neurospora*, *Gonyaulax* and, more recently, cyanobacteria and mammals), we appreciate the enormous complexity of the circadian programme in organisms and cells, as well as in physiological and molecular circuits. Many of our insights into this complexity stem from experimental reductionism that goes as far as testing the interaction of molecular clock components in heterologous systems or *in vitro*. The results of this enormous endeavour show circadian systems that involve several oscillators, multiple input pathways and feedback loops that contribute to specific circadian qualities but not necessarily to the generation of circadian rhythmicity. For a full appreciation of the circadian programme, the results from different levels of the system eventually have to be put into the context of the organism as a whole and its specific temporal environment. This review summarizes some of the complexities found at the level of organisms, cells and molecules, and highlights similar strategies that apparently solve similar problems at the different levels of the circadian system.

Keywords: circadian; model; *Neurospora*; feedback; transcription; *Zeitnehmer*

1. INTRODUCTION

At one end, circadian complexity involves the organism's entire physical and biological environment—with all participants (rotation of earth, food, predators, etc.) somehow being oscillators. At the other extreme, a set of genes forms a feedback loop. A strategy to cope with complexity is to reduce the number of variables in experiments and to control those remaining. As a first step in circadian research, organisms were isolated from their environment—subjected to only one *zeitgeber* or to constant conditions. Fundamental findings resulted from this approach: rhythmicity continues unabated in constant conditions, it can be entrained, in defined ranges, to 24-hour and non-24-hour cycles (e.g. by using light as the *zeitgeber*), and it appears to defy biochemical logic by compensating its period for different constant temperatures. These characteristics can be broken down into the following six qualities which are common to all circadian systems (Roenneberg & Merrow 1998):

(i) Rhythmicity as such (independent of its frequency).
(ii) The circadian range of the period.
(iii) An amplitude sufficiently robust to drive output rhythms.
(iv) The fact that the rhythmicity is sufficiently self-sustained to continue unabated.
(v) Temperature compensation.
(vi) Entrainability.

Despite our definition of circadian systems and their qualities, many important questions remain unanswered. Why, for example, is circadian rhythmicity potentially undamped, although the clock would get its daily kick in nature? What is the function of temperature compensation? Perhaps the solutions to these questions are rooted in the system's complexity both at the anatomical and molecular level.

The question of where the clock resides in animals has been approached by ablation and transplantation experiments (e.g. Richter 1967; Ralph *et al*. 1990). Ablation of identified pacemakers, e.g. the suprachiasmatic nuclei (SCN) results in loss of rhythmicity and their transplantation rescues rhythmicity together with other circadian qualities. Yet even without these pacemakers, organisms can still consolidate circadian behaviour under special conditions. Other oscillators must therefore remain, but what are they and why are there so many?

After isolating the organism from its 'noisy' environment, central pacemakers can be isolated from the 'noisy' rest of the organism and still maintain their circadian properties *in vitro*. Even when individual pacemaker cells are separated, they continue to generate circadian rhythmicity. However, the properties of the single cell rhythmicity are different from those of the network. As independent cells, they oscillate with different periods and out of phase (Welsh *et al*. 1995). Networks are therefore important for uniform and robust rhythmicity of the organism but not for the generation of the oscillation, which is cellular. Furthermore, self-sustained cellular oscillation is not confined to pacemaker cells—essentially the same molecular clocks appear to tick in cells of different tissues, from brain to liver in mammals and fish (Whitmore *et al*. 2000; Yamazaki *et al*. 2000), from head to wings in *Drosophila* (Plautz *et al*. 1997). Like in the ecosystem, chains of oscillators form networks and potentially feed back on or modulate each other within an organism.

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Experimental verification of 'clock gene' function has been approached in analogous ways to experiments at the anatomical level, i.e. deletion and re-introduction of a 'clock gene' go together with loss and rescue of rhythmicity, with circadian qualities linked to a gene (Dunlap 1999). The complexity problem also extends analogously from the anatomical to the cellular–molecular level—without a 'clock gene', an entrainable oscillator remains (Merrow *et al*. 1999) and single cells may even accommodate more than one circadian oscillator (Roenneberg & Morse 1993). So, the circadian complexity known from the higher levels can even be demonstrated within the cell.

In spite of all this reductionism, complexity reappears, although the players change—organisms, cells, molecules. By comparing strategies at the different levels, we learn how (and possibly why) elements are put together. By characterizing more of the players and discovering how they interact, we will finally understand how the system works as an entity.

2. CIRCADIAN COMPLEXITY WITHIN THE ORGANISM

The mammalian pacemaker resides in the SCN (figure 1) and drives the rhythmic melatonin production in the pineal—but, unlike in many other vertebrates, the production of melatonin is not rhythmic in the isolated pineal. The fact that pineal melatonin production is driven by the SCN in mammals does not mean that mammalian pinealocytes do not contain a cellular–molecular circadian clock, as do many other cells and tissues (Yamazaki *et al*. 2000). Recent experiments indicate that 'clock gene' RNAs also cycle in isolated pineals (S. Yamazaki, M. Abe, E. D. Herzog and M. Menaker, personal communication; G. Tosini, personal communication). Thus, pinealocytes apparently contain a molecular clock—only their melatonin production depends on SCN control via a multisynaptic pathway. The SCN as a circadian structure did not first appear with the evolution of mammals as non-mammalian vertebrates also possess a hypothalamic circadian clock, so that at least two autonomous pacemakers coordinate their endogenous daily programme (we know the most the about their interplay from experiments with sparrows, e.g. Takahashi & Menaker 1979; Takahashi *et al*. 1980; Heigl & Gwinner 1999). The respective role of different clock centres in the non-mammalian circadian programme appears to have an important function in fine-tuning physiology, morphology and behaviour in different seasons and in different life-stages (Gwinner 1986; Foà *et al*. 1994; Innocenti *et al*. 1996; Menaker & Tosini 1996; Gwinner & Brandstätter 2001). The mammalian pineal with its melatonin production not only plays an important role in transducing the signal of external darkness throughout the body but it also forms a feedback loop with the SCN, thereby contributing to the functioning of the circadian system itself (figure 1; Steinlechner 1989; Cassone 1992; Cassone *et al*. 1993; Gillette & McArthur 1996; Weaver & Reppert 1997; Von Gall *et al*. 1998; Masana *et al*. 2000). The eyes, too, have independent circadian oscillators (Tosini & Menaker 1996) and also produce melatonin (Herzog & Block 1999), but little is known about the role of the retinal clock in the entire system. This is also true for other, as yet unidentified, circadian centres that are postulated, based on experiments in SCN-lesioned rats (e.g. amphetamine-dependent activity rhythms, Honma *et al*. 1987). In addition, pacemaker centres must exist in parallel to the SCN-based circadian system. An anticipatory locomotor behaviour in rodents can be entrained by food without entraining the SCN-controlled activity rhythm (Aschoff 1987). The characteristics of this food entrainable oscillator (FEO) are similar to but separate from the circadian system.

Mainly based on monitoring mRNAs of known 'clock genes' (Balsalobre *et al*. 1998) or by using light-emitting reporter constructs combining clock-controlled promoters and a luciferase gene, many autonomous circadian clocks have been discovered outside of the brain in insects and vertebrates (Hege *et al*. 1997; Plautz *et al*. 1997; Whitmore *et al*. 2000; Yamazaki *et al*. 2000; Giebultowicz 2001). In spite of the identification of molecular circadian clocks in many mammalian peripheral tissues and cells, the role of the SCN as a central pacemaker is unchallenged (Yamazaki *et al*. 2000). The different body clocks have different qualities with fundamental differences between the molecular clock of SCN neurones and those of peripheral cells. While a cultured SCN oscillates without damping as long as the cells can be kept alive in culture, peripheral oscillators damp within one or two cycles (Yamazaki *et al*. 2000). Damping is prevented when the culture medium is regularly refreshed, suggesting that the peripheral molecular oscillators have to be regularly re-initiated (or re-entrained among each other). There are at least two possible explanations for the differences between cellular clocks in the pacemaker and in the periphery. Either the molecular machinery responsible for their circadian rhythmicity is put together differently or SCN neurones produce output signals (which can feed back and sustain the oscillation) while peripheral cells only organize their own metabolism on a circadian scale without exporting circadian signals. The issue of feedback and self-sustainment will be discussed in more detail below (see § 4).

Clocks are thought to have evolved because entrainment of an oscillator apparently allows more flexibility than merely being driven by the cyclic environment (Pittendrigh 1960; Yan *et al*. 1998). The same argument holds for units within the organism. The temporal organization of cells and tissues should also be more flexible when they are entrained, in this case by an endogenous *zeitgeber*. Thus, the SCN is entrained to exogenous *zeitgeber* (e.g. light) and produces (directly or indirectly) endogenous *zeitgebers* to entrain the peripheral target clocks. Endogenous *zeitgeber* time can either be transmitted via neuronal pathways (Buijs *et al*. 1999) or via factors, such as glucocorticoids, circulating in the bloodstream (Balsalobre *et al*. 2000). As such, clocks within animals constitute a clearly structured hierarchy similar to those in ecosystems.

This hierarchy apparently does not exist in higher plants. Different parts of the plant can be entrained independently by different light–dark cycles (e.g. the tip of the leaf to San Francisco and the rest to Edinburgh time). These unnatural phase relationships are even maintained when the plant is exposed to constant light (Thain *et al*. 2000). No communicating agents appear to couple

the different cellular plant clocks. This is surprising because plants, like animals, have to coordinate different anatomical parts in many ways (e.g. the regulation of turgor), and temporal regulation is probably not exempt from this coordination. Synchrony of the different clocks would be achieved if each part of the plant was perfectly entrained by the environment. Another scenario is a circadian programme composed of more than one clock system: one ticking autonomously and independently in every cell and another capable of endogenous coupling, which is responsible for the coordination of different anatomical parts over the course of a day. The latter is not unlikely because independent circadian oscillators have already been shown to exist in a unicellular alga (Roenneberg & Morse 1993).

3. CIRCADIAN COMPLEXITY WITHIN THE CELL

A cellular, rather than network-based, generation of circadian rhythmicity has been known for several decades based on work with single cell organisms, mainly algae (Pohl 1948; Hastings & Sweeney 1958; Sweeney 1987; Edmunds 1988). The marine dinoflagellate, *Gonyaulax polyedra*, like other unicellular organisms (Edmunds 1988), generates the entire circadian programme, from metabolism to behaviour, within a single cell. During the day, the cells aggregate in the upper layer of the ocean exploiting sunlight for photosynthesis, whereas during the night they sink to lower layers and take advantage of the higher concentrations of fixed nitrogen. Both light and nitrogen are not only resources for *Gonyaulax*, but act also as *zeitgeber* for its circadian system. Light reaches the clock via at least two separate light input pathways (LIPs) (Roenneberg & Hastings 1988; Roenneberg & Taylor 1994; Roenneberg & Deng 1997). One of the LIPs is predominantly sensitive to blue light and is activated only during the night, forming an input feedback (see figure 2). The other LIP responds both to red and blue light, possibly via photosynthesis, which is itself an output of the clock. Thus, photosynthesis also forms a (metabolic) feedback loop. The same is true for nitrogen metabolism (Ramalho *et al*. 1995; Roenneberg & Rehman 1996) and regulation of pH (B. Eisensamer and T. Roenneberg, unpublished data; Hastings 1960). Thus, the cellular circadian system of *Gonyaulax* consists of multiple feedback loops beyond the one that generates the rhythmicity (rhythm generator, figure 2). To add to this complexity, *Gonyaulax* regulates its temporal programme with the help of at least two circadian oscillators (Roenneberg & Morse 1993), which control different parts of metabolism and different aspects of behaviour, respectively, and respond differently to environmental signals.

One explanation for this enormous complexity within the *Gonyaulax* circadian system lies in the fact that all aspects of the temporal programme must be implemented within a single cell. This does not necessarily predict that the cellular clocks in higher organisms are similarly complex. Like many other functions in higher animals and plants, the cellular clock systems could be subject to specialization. There are, however, indications that the molecular machinery of the cellular circadian system in higher organisms may also be more complex than a single (Hardin *et al*. 1990; Aronson *et al*. 1994*b*) or multiple (Glossop *et al*. 1999; Lee *et al*. 2000; Shearman *et al*. 2000) molecular feedback loops. A case in point is found in the filamentous fungus *Neurospora crassa*.

The circadian formation of aerial hyphae and conidia (or asexual spores) is used as the clock's read-out in *Neurospora*. Molecular components that are central to the molecular circadian machinery in *Neurospora* are the genes *frequency* (*frq*), *white collar 1* (*wc-1*) and *white collar 2* (*wc-2*) (Dunlap 1999; Bell-Pedersen *et al*., this issue). Several *frq* alleles have been isolated or engineered (including short and long period mutants, as well as those resulting in apparent arrhythmicity in constant conditions (Feldman & Hoyle 1973; Loros & Feldman 1986; Aronson *et al*. 1994*a*; Dunlap 1996)). FRQ and the regulation of its transcription form an autoregulating negative feedback that is essential for normal rhythmicity (Aronson *et al*. 1994*b*). WC-1 and WC-2 are the positive elements (activating, directly or indirectly, *frq* transcription) and FRQ is the negative component in the feedback, though it also contributes a positive effect on WC-1 protein (Lee *et al*. 2000) and RNA levels (Merrow *et al*. 2001). However, more molecular components have to be postulated to participate in the generation of circadian rhythmicity in *Neurospora*. The two functional null mutants, frq^{9} and frq^{10}, are not always arrhythmic; after several days in constant darkness (DD), growing on certain media, rhythmic conidiation will sometimes appear (Loros & Feldman 1986; Aronson *et al*. 1994). Although they are conditionally arrhythmic in DD and not entrainable by light (Chang & Nakashima 1997; Merrow *et al*. 1999; Lakin-Thomas & Brody 2000), the null mutants remain entrainable in the circadian range by temperature cycles (Merrow *et al*. 1999; Roenneberg & Merrow 2001). Thus, an as yet uncharacterized oscillator controls conidiation in the absence of a functional FRQ protein. This reopens the question about the exact function of FRQ in the *Neurospora* circadian system.

The observation that circadian input pathways are often under clock control (for a review, see Roenneberg & Merrow 2000), thereby forming a feedback loop between the rhythm generator and its input (figure 2), led us to 'ask' computer models whether rhythmic elements of an input pathway to the clock (domains II and III in figure 2) and components within the rhythm generator (domain I, figure 2) can be discriminated experimentally (Roenneberg & Merrow 1998). The results show that elements in both locations can lead to phenotypes that have traditionally been assigned only to mutations of elements within the rhythm generator. The traditional view of a one-way input pathway, a rhythm-generating loop, and unidirectional output pathways has been an excellent scheme for finding critical components within the circadian pathway at both the anatomical and the molecular level. However, many recent experimental results, as well as the theoretical results of modelling, demand a revision of this view to a more complicated scheme that discriminates between different domains of the system (figure 2).

But how can one distinguish between the location of different elements if phenotypes of their mutants can be so similar? Although malfunction in all domains of the system can lead to arrhythmicity (except for unidirectional outputs in domain IV), the clock still should retain some of its properties if domain I is still intact. If the

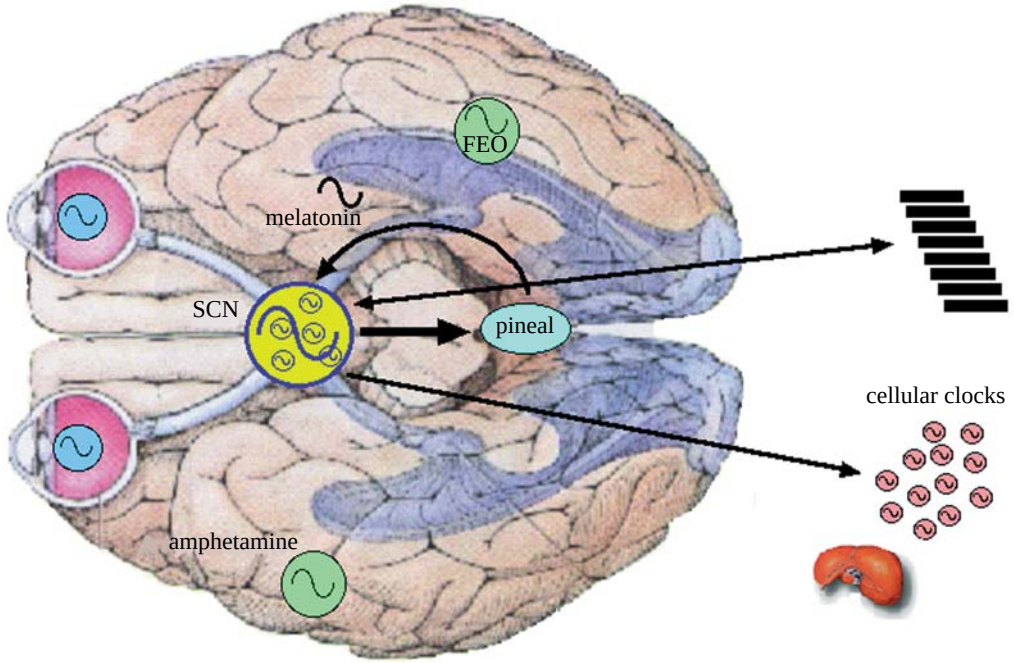

Figure 1. Circadian complexity within the organism (demonstrated by the mammalian system). The central pacemaker resides in the suprachiasmatic nuclei (SCN) but isolated SCN neurons are still able to produce circadian rhythmicity. Rhythmic melatonin production in the pineal depends on the SCN but also feeds back to influence the hypothalamic clock. The eyes also contain independent circadian clocks. Generally, the SCN is responsible for circadian behaviour but consolidated circadian activity rhythms can be recorded in SCN-lesioned rats when given amphetamine. The multiple clocks in peripheral cells, tissues and organs probably depend on an intact SCN and its output signals (internal *zeitgeber*) to oscillate in coordination. Entrainment by the external *zeitgeber* light relies in mammals on the retinohypothalamic tract, i.e. on intact eyes and SCN. In parallel to the SCN based oscillator, there is another food entrainable oscillator (FEO) with similar characteristics (e.g. anticipation and specific phase angles in the entrained state). Animals rendered arrhythmic by ablation of the SCN can exhibit circadian rhythmicity in their activity when supplied with amphetamines (see text and Honma *et al*. 1987).

mutation is, for example, in the light input, the system will lose its entrainability to light but not necessarily to other *zeitgeber*, as is the case for the *Neurospora* 'clock gene' *frq* (Merrow *et al*. 1999, 2001). All known and new 'clock genes' have to be eventually investigated for their location within the system. Entrainment by different *zeitgebers* (e.g. by both light and temperature) should be routinely part of the experimental protocols describing the circadian function of a putative 'clock gene'. Table 1 summarizes the known 'locations' of 'clock genes' within the circadian system on the basis of the domains shown in figure 2. The list is far from complete, and we have assigned the location of the components according to how the genes are described in the literature. With further discriminating experiments, as described above, these placements will surely change over time. In some cases, assignment is relatively certain, e.g. mutants of several plant genes (such as *TOC1* or *PHYB*) change period and/or light responsiveness without disrupting self-sustained rhythmicity in constant conditions. In other cases assignment is difficult. WC-2, for example, is non-rhythmic (both RNA and protein) but forms complexes with other rhythmic components (FRQ and WC-1: Talora *et al*. 1999; Denault *et al*. 2001; Merrow *et al*. 2001).

4. COMMON PROBLEMS AND SOLUTIONS

The circadian systems of whole organisms and those of cells share features of complexity. Both can use more than one oscillator and both engage feedback loops outside of the pacemaker or rhythm generator, respectively. One possible approach to understanding the systems on both levels is to consider the problems with which they have to cope.

Circadian systems can be regarded as pathways (figure 2) with a sensory function at one end (sensing external or internal signals, *zeitgeber* and *zeitnehmer*) and control functions at the other end (the output rhythms), with circadian timing added somewhere along the way. At all biological levels, from neuronal networks to molecules, sensory functions are highly regulated and most of them actively 'probe' for signals rather than respond to them passively. They are capable of complex signal processing (e.g. adaptation) and can be regulated by other systemic functions (e.g. by memory, fatigue, other sensory modalities at organismal level, or by the metabolic state on the cellular level). Due to their elaborate processing, sensory input pathways are not just 'silent' when stimuli are absent. One common problem for sensory functions is the discrimination between signal and noise. Circadian systems have to cope with two sources of noise: external and internal. One of them is connected with detection of external signals. For the organism, the *zeitgeber* light, for example, contains 'noise' introduced by clouds and behaviour. The same applies to endogenous *zeitgebers*: factors in the bloodstream or neurotransmitters are also challenged by the 'noise' of metabolism and physiological functions.

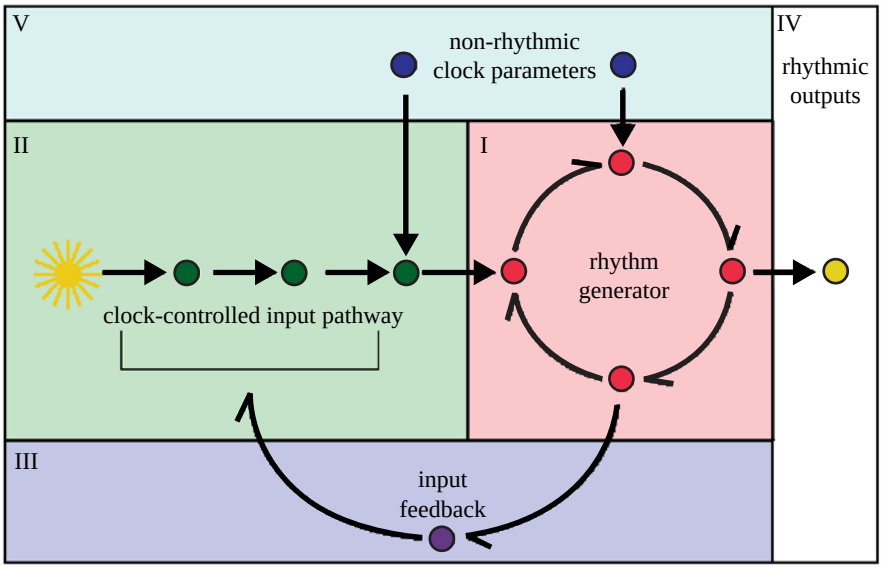

Figure 2. Circadian complexity within the cell. At the heart of a circadian system, a mechanism generates the rhythmicity, possibly via some negative feedback loop (domain I; note, this generator on its own can theoretically produce a rhythm outside of the circadian range, e.g. 12 h; the circadian period can be tuned by other components of the system). For entrainment with the 24-hour day, input pathways (domains II or V) transduce environmental information (*zeitgeber*, time giver) that resets elements of the rhythm generator. Input pathways can themselves be under circadian control (domain II) via a feedback from the rhythm generator (domain III; *zeitnehmer* = time taker, see text). *Zeitnehmer* loops influence period length and robustness of the oscillating system. The distinction between elements in domains I, II and III is difficult (see also table 1)—the scheme still shows them as separate because, theoretically, clock-controlled elements in domain II should respond to light (or other *zeitgeber*) directly, whereas those in domain III should only respond via shifting the phase of the rhythm generator. Any deletion in a *zeitnehmer* loop (domains II and III) may render the entire system arrhythmic. Deletions of elements in domain II make the system unresponsive to one *zeitgeber* and not necessarily to others, while those in domain III will make the respective input pathway non-rhythmic. All components of rhythm generators and *zeitnehmer* loops as well as of outputs (domain IV) are rhythmic. Although they are here drawn as originating from the rhythm generator, they could theoretically be controlled by any rhythmic element of the system. In addition to the rhythmic components, other non-rhythmic elements (including those of a non-rhythmic input pathway) can be essential for circadian function (domain V). (Figure redrawn from Roenneberg & Merrow 2000.)

An excellent example of how circadian systems cope with internal noise is temperature compensation. Although the rate of metabolism and of individual enzymes changes with temperature, the circadian period does not. Interestingly, several mutations of 'clock genes' in different organisms have also lost temperature compensation (see also Morgan *et al.*, this issue). The circadian clock of *Neurospora*, for example, runs faster with higher temperature in several *frq*-mutants (e.g. in frq^9, frq^{10}, and frq^7). Thus, FRQ has at least three functions in the *Neurospora* clock: (i) when the negative feedback by FRQ on *frq* transcription is defective in a mutant, rhythmicity in constant darkness appears only under special conditions (e.g. in frq^9, frq^{10}); (ii) when the protein is altered by a mutation, with negative feedback still intact, rhythmicity in DD continues (though with a different period), although temperature compensation is partially lost (e.g. in frq^7); (iii) mutations in *frq* can change the sensitivity of the clock to light (e.g. in frq^1-increased sensitivity and in frq^7-decreased sensitivity: Dharmananda 1980; Lakin-Thomas *et al.* 1991), and without functional FRQ the clock becomes unresponsive to light (e.g. in frq^9, frq^{10}). All these different functions of FRQ can be best explained if this 'clock gene' product is placed in a clock-controlled input pathway (domain II in figure 2) (Merrow *et al.* 1999, 2001).

But what is the purpose of circadian input feedbacks? One of the functions is simply to probe actively for a signal and for modulation of the strength of a given physical or chemical stimulus according to circadian time. It is interesting that most *zeitgeber* signals have the greatest effects on the clock at a time when they would be absent or lowest in the daily cycle of the natural environment. For example, light is most effective in the subjective night in all organisms (Roenneberg & Foster 1997) or nitrate is most effective during the subjective day in *Gonyaulax*, when the cells normally are found in the low nutrient upper layers (Roenneberg & Rehman 1996). In other words, *zeitgeber*s are most effective when a subjective time is exposed to an environmental signal at the 'wrong' time. In analogy with the term *zeitgeber* (time giver), we have therefore called the process of actively probing for time cues '*zeitnehmer*' (time taker) (Roenneberg *et al.* 1998; Lakin-Thomas 2000).

However, *zeitnehmer* loops, being both input and output of the rhythm generator (figure 2), may have other important functions in the circadian system. Modelling the effect of *zeitnehmer* loops on circadian rhythmicity in constant conditions shows that they affect period length and robustness of the rhythm. In addition, they also contribute to decreasing the effect of noise. When the rate constants of every 'reaction' of the model are submitted to the same simulated noise (random fluctuations), the robustness of the system's rhythmic output (judged by the regression coefficient of autocorrelation) decreases rapidly

Table 1. Localization of 'clock genes' within the circadian system.

(Based on the domains shown in figure 2. Some assignments reflect how the respective genes are presently assigned in the literature without having been submitted to experiments discriminating between the different possibilities. References are kept to a minimum, although many other papers could have been cited that describe the function of the respective gene in the system.)

	domain					
	I	II	III	IV	V	reference
plants (*Arabidopsis*)						
TOC1		•	•			Strayer *et al.* (2000)
FKF1		•	•			Nelson *et al.* (2000)
ZTL					•	Somers *et al.* (2000)
GI		•	•			Park *et al.* (1999)
CRY1		•				Somers *et al.* (1998)
CRY2		•				Somers *et al.* (1998)
PHYB		•				Bognar *et al.* (1999), Somers *et al.* (1998)
PHYA		•				Somers *et al.* (1998)
ELF3		•	•			A. Millar (personal communication)
CCA1	•					Wang & Tobin (1998)
LHY	•					Schaffer *et al.* (1998)
CK2					•	Sugano *et al.* (1999)
CCR/AtGRP7				•		Heintzen *et al.* (1997)
mammals						
per1	•	•				Shigeyoshi *et al.* (1997)
per2	•	•				Albrecht *et al.* (1997)
per3	•					Takumi *et al.* (1998)
bmal1	•					Darlington *et al.* (1998)
clock					•	Gekakis *et al.* (1998)
dbt (*tau*)					•	Lowrey *et al.* (2000)
cry1	•					Kume *et al.* (1999)
cry2	•					Kume *et al.* 1999)
vasopressin gene (e-box)				•		Jin *et al.* (1999)
Drosophila						
per	•					Hardin *et al.* (1990)
tim	•	•				Hunter-Ensor *et al.* (1996), Sehgal *et al.* (1995)
dbt					•	Price *et al.* (1998)
cyc (*bmal*)	•					Rutila *et al.* (1998)
jrk (*clock*)	•					Allada *et al.* (1998)
cry		•	•			Ceriani *et al.* (1999), Emery *et al.* (1998), Stanewsky *et al.* (1998)
lark				•		McNeil *et al.* (1998)
Neurospora						
frq	•	•				Aronson *et al.* (1994*b*), Merrow *et al.* (1999)
wc-1	•	•				Crosthwaite *et al.* (1997), Lee *et al.* (2000), Merrow *et al.* (2000)
wc-2					•	Dunlap (1999)
all ccgs				•		Bell-Pedersen *et al.* (1996)
Synechococcus						
KaiA	•					Ishiura *et al.* (1998), Xu *et al.* (2000)
KaiB	•					Ishiura *et al.* (1998), Xu *et al.* (2000)
KaiC	•					Ishiura *et al.* (1998), Xu *et al.* (2000)
SasA		•	•			Iwasaki *et al.* (2000)
CikA		•				Schmitz *et al.* (2000)
Cr-1		•				cf. Iwasaki & Kondo (2000)
CpmA				•		cf. Iwasaki & Kondo (2000)
RpoD2				•		cf. Iwasaki & Kondo (2000)

when no *zeitnehmer* loop is closed (figure 3*a*, thin curve) but is less susceptible with a closed input–output loop (figure 3*a*, thick curve; the mathematics of these models are described in detail in Roenneberg & Merrow 1999). Similarly, if the rate constants are increased (mimicking, for example, different temperature levels), the period is shortened with increasing rates when the system does not contain a *zeitnehmer* loop (figure 3*b*, thin curve); otherwise the period is compensated over a wide range of different (theoretical) reaction rates (or temperatures; figure 3*b*, fat curve). Temperature compensation in circadian systems has been interpreted by analogy with temperature compensation in mechanical clocks (what use is a clock that changes with different ambient temperatures?). Yet,

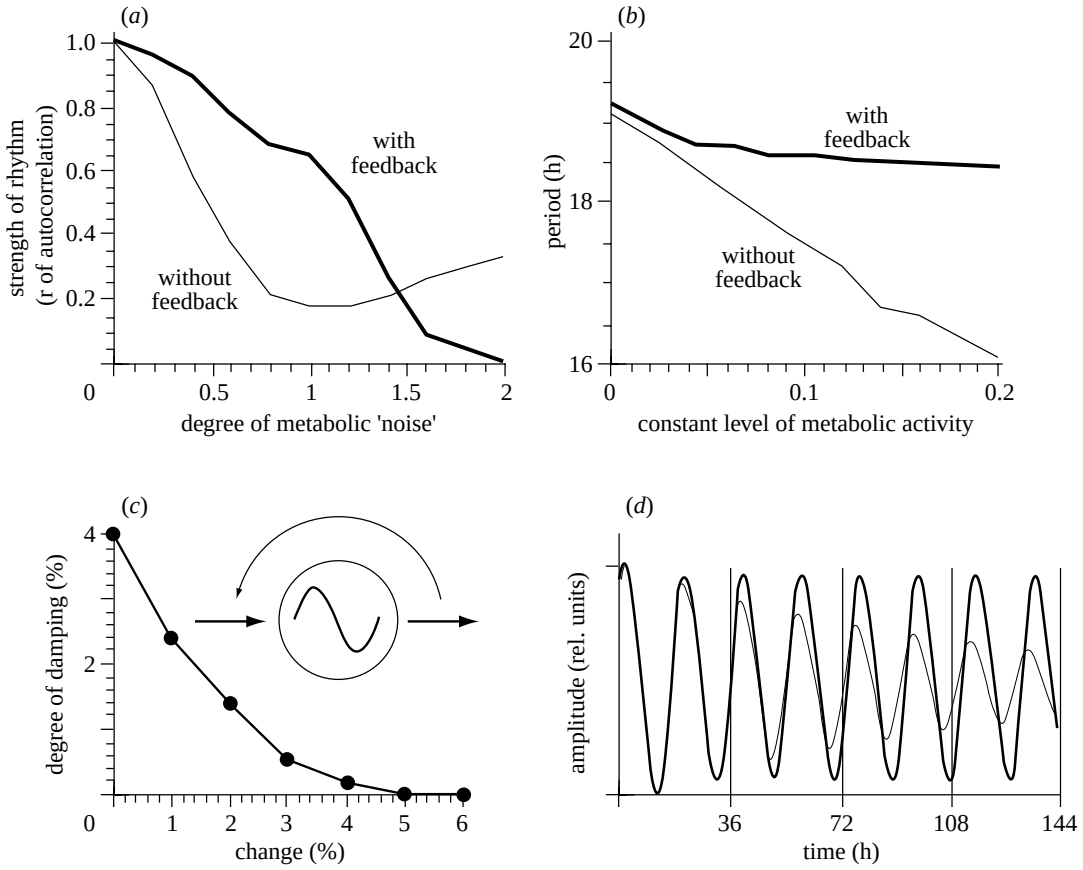

Figure 3. Effects of *zeitnehmer* loops on the circadian system. Theoretical modelling shows that input feedbacks, as shown in figure 2 (domains II and III), affect the period length and the robustness of the clock (the mathematics of the computer models shown in this figure are described in detail elsewhere: Roenneberg & Merrow 1999). In addition to their effects on period and robustness, feedback loops reduce the susceptibility of the clock to (metabolic) noise (*a*) and contribute to the compensation of the clock to different levels of (metabolic) activity, e.g. temperature compensation (*b*). They can even be the basis of self-sustainment of circadian rhythmicity in constant conditions (*c* and *d*); depending on the strength of the *zeitnehmer* feedback, a damped oscillator becomes increasingly self-sustained. The damped oscillator shown in panel *c* becomes self-sustained (*d*) when the feedback changes the 'current' along the input by 5% (analogous to an eyelid shielding off 5% of the light when closed and none when open). Thus, self-sustainment of circadian rhythmicity could be a consequence rather than a prerequisite of the circadian system.

the circadian period is not only compensated for different temperatures. The circadian clock also compensates for a period over a large range of different light intensities as well as for different nutrient concentrations. Thus, temperature compensation may be only one aspect of metabolic compensation (Feldman & Stevens 1973; Pittendrigh *et al*. 1973) and *zeitnehmer* loops could contribute to shielding the period against different constant levels and noise. This possibility is supported by findings in the *Neurospora* circadian system. The fact that the wild-type strains are rhythmic with a similar period on all media and over a wide range of temperatures, while the null mutants are rhythmic only under special nutritional conditions, indicates that mutations in 'clock genes' can affect both temperature and metabolic compensation (Loros & Feldman 1986; Roenneberg & Merrow 1999).

If the parameters of a computer-modelled rhythm generator are chosen so that its amplitude damps over time (figure 3*c*), an input–output feedback loop can make the rhythmicity self-sustained. With increasing strength of the feedback, the rhythm's amplitude damps at a slower rate. If the clock-controlled feedback changes the 'dark current' transduced by the input pathway (i.e. without external stimulation) by only 5%, the rhythm becomes self-sustaining (figure 3*d*).

Input–output feedbacks do not have to be wired into the molecular components of an input pathway. If, for example, cells produce circadian output signals (as SCN neurones surely do) that can feedback onto the circadian clock of the cell, then the result could be self-sustainment. If a cellular circadian system, however, is the endpoint of a circadian hierarchy (e.g. as in peripheral tissues and cells), circadian output signals may not be produced and rhythms might damp without the regular signals of circadian factors (Yamazaki *et al*. 2000). Similar arguments have been used to explain the precision and self-sustainment of circadian rhythms in populations of unicellular organisms in experimental 'captivity' (Roenneberg & Mittag 1996). *Gonyaulax* cultures prime their artificial environment in small containers in a circadian fashion and, at the same time, respond to components in the culture medium. Input–output feedbacks may even contribute to self-sustainment at the behavioural level. Activity and rest, wakefulness and sleep are circadian outputs and, at the same time, can affect the circadian clock, either via non-photic pathways

involving different levels of motor activity (Reebs & Mrosovsky 1989) or by modulating exposure to constant light through behaviour (e.g. closing eyelids, Boivin *et al*. 1994). Behavioural feedbacks could even explain why SCN-less rats consolidate a circadian activity pattern with amphetamines (Honma *et al*. 1987); increased activity levels and the concurrent metabolic changes could help to synchronize the peripheral clocks.

Zeitnehmer feedbacks could account for many features of the circadian clock without themselves generating the oscillation. The scheme shown in figure 2 represents feedbacks within the molecular circuit of the cellular circadian system. It could, however, also be used to describe circadian circuits on higher levels of the organism. In this case, the pathway would contain many circadian oscillators, e.g. ranging from those in the eyes via the SCN (as the main rhythm generator for the entire system) to cellular circadian clocks in the target tissues. The notion of master and slave oscillators within a circadian pathway goes back to Pittendrigh (1981) as well as the hypothesis that several coupled but largely independent oscillators (E and M=evening and morning) are more efficient in measuring complex changes of the environmental day over the course of the year (Pittendrigh & Daan 1976). The concept of E and M oscillators has recently been revisited in reference to cellular and molecular clocks (Daan *et al*. 2001; Jagota *et al*. 2000).

Similar strategies are therefore found at different levels of the system. Although they may be implemented by different units (e.g. cells and molecules), they are designed to cope with similar problems, e.g. external and internal noise, measuring the length of the day, or controlling different circadian outputs with a high flexibility. The identification of problems and coping strategies at the different levels of the system may facilitate seeing the circadian 'wood' for the circadian 'trees'.

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