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Circadian Systems and Metabolism

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Abstract Circadian systems direct many metabolic parameters and, at the same time, they appear to be exquisitely shielded from metabolic variations. Although the recent decade of circadian research has brought insights into how circadian periodicity may be generated at the molecular level, little is known about the relationship between this molecular feedback loop and metabolism both at the cellular and at the organismic level. In this theoretical paper, we conjecture about the interdependence between circadian rhythmicity and metabolism. A mathematical model based on the chemical reactions of photosynthesis demonstrates that metabolism as such may generate rhythmicity in the circadian range. Two additional models look at the possible function of feedback loops outside of the circadian oscillator. These feedback loops contribute to the robustness and sustainability of circadian oscillations and to compensation for long- and short-term metabolic variations. The specific circadian property of temperature compensation is put into the context of metabolism. As such, it represents a general compensatory mechanism that shields the clock from metabolic variations.

Key words oscillator models, circadian system, metabolic feedbacks, temperature compensation

Over the past few years, circadian research has taken off. The molecular biology of the circadian clock is certainly the driving force of this incredible surge. More and more molecular components are being discovered that play an intimate role in generating or regulating circadian oscillators; nonetheless, many fundamental questions of circadian biology remain unanswered in the context of the temporal ecology of whole cells and intact organisms.

One of the longstanding questions in circadian biology concerns the involvement of metabolism in circadian rhythmicity. To date, it is not clear whether metabolism is only an output of the clock or whether it also contributes to the rhythm's generation (beyond providing the necessary cellular energy). Circadian rhythmicity in metabolic functions and metabolites (photosynthesis, redox, energy charge, pH, Ca^{2+} -concentrations, etc.) has been extensively demonstrated, mainly in algae, microorganisms, and higher plants (Brody, 1992; Byrne et al., 1992; Delmer and Brody,

1975; Johnson, 1992; Johnson et al., 1995; Mattern and Brody, 1979; Wagner et al., 1984). On the other hand, several metabolic mutants were found to result in altered circadian properties (Coté, 1987; Lakin-Thomas, 1998).

There are many indications that metabolism is not just an output of the circadian clock but is involved in its control. First, heavy water (D_2O), which affects many metabolic reactions, uniformly slows down all circadian systems tested so far (Bruce and Pittendrigh, 1960; Daan and Pittendrigh, 1976). However, its effects on circadian rhythms appear to be distinct from the effects of decreasing temperature (McDaniel et al., 1974; Pittendrigh et al., 1973). Second, one of the early methods of showing rhythmicity within the SCN in vivo was based on circadian changes in neuronal metabolism, as determined by the uptake of radioactive deoxyglucose (Schwartz and Gainer, 1977). Third, the nonphotic (feedback) effects of activity onto the circadian system in mammals most likely involve

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highly increased metabolic rates as they are mainly associated with excessive wheel-running activity (Mrosovsky, 1988). Similarly, the effects of the benzodiazepine triazolam on the circadian system of rodents (Turek and Losee-Oldson, 1986) are also associated with a large increase of motor activity (Turek, 1989; Wickland and Turek, 1991b). These effects have been proposed to act through activity rather than through pharmacological effects on the clock itself (Reebs and Mrosovsky, 1989). These circadian effects, however, do not correlate with changes in body temperature (Wickland and Turek, 1991a). Activity can even consolidate a circadian activity rhythm in rodents after removal of the SCN. When amphetamine is given to these SCN-lesioned arrhythmic animals, their activity level is greatly increased and consolidated in circadian episodes (Honma et al., 1986, 1991). Lastly, internal desynchronization between the temperature and the activity rhythm, which can be observed in some freerunning humans is also linked to metabolism (Aschoff, 1965; Aschoff et al., 1967). During long desynchronization, when subjects can live up to 50-h days, they eat the same amount of food as they do in a 24-h day also distributed over three meals without losing weight (Aschoff, 1995). However, their basal metabolism appears to be decreased during this long desynchronization period (Aschoff, 1994). Yet, the body core temperature rhythm continues to oscillate with a period around 25 h and, thus, appears to be compensated for the metabolic change. Thus, metabolism and different activity levels can influence the circadian oscillator independent of associated temperature changes.

A nother pertinent question of circadian research asks whether the basic mechanisms of the clock have evolved once and have been passed down the phylogenetic tree or whether they originated and evolved (analogously) several times in evolution. The homology of certain molecular components in insects compared with mammals suggests a single origin with conservation of circadian mechanisms within the animal kingdom. The findings in cyanobacteria, fungi, and plants have so far not provided evidence for a wider homology (except for the photolyase/ cryptochrome family). But even homologous circadian components, for example, between flies and moths or insects and mammals, can have different molecular fates, perhaps reflecting species-specific differences in temporal ecology and metabolism. The species-specific role of these transcriptional feedback loops in

the circadian system may become apparent when we understand their relationship to cellular metabolism.

In the unicellular alga *Gonyaulax*, cellular metabolism is closely linked to the circadian system. Energy (from light) and nutrients are spatially separated in the water column. During the day, *Gonyaulax* cells aggregate in the top layer of the ocean and photosynthesize; during the night, they sink to the bottom and exploit the higher concentrations of fixed nitrogen. So, behavior and metabolism are coordinately controlled by the circadian program to optimize access to light and nitrate. However, these essential resources can also act as zeitgeber for *Gonyaulax*. Manipulations of these resources and their metabolizing enzymes demonstrate the effects of metabolism on circadian rhythms, changing their amplitude, phase, and period. That metabolic pathways are both under circadian control and function as input pathways to the circadian system shows that feedbacks exist outside of a central oscillator. In this paper, we try to integrate the central rhythm generator, additional feedback loops, and the daily changes in metabolism.

METHODS

All models have been implemented with the help of the Stella Program (Stella Research, Hanover NH, version 5.1.1. for Power Macintosh). Iteration rate was set to 30 min in all model runs. The resulting rhythms were analyzed with the help of the CHRONO program (Roenneberg and Taylor, 1999).

Model 1: Photosynthesis Feedback

CO_2 -concentration (initial value is 50) depends at any given time (t) on respiration (producing CO_2 at a constant rate of 2) and its depletion by carbon fixation (CF) as a result of photosynthesis (a diagram of this model is shown in Fig. 1).

$$CO_2(t) = CO_2(t - dt) + (2 - 0.5 * CF) * dt \quad (1)$$

Carbon fixation (CF, initial value is 10) depends on the rate of photosynthesis (r_{PS}) and its outflow into further anabolic pathways (with a constant rate of 9).

$$CF(t) = CF(t - dt) + (r_{PS} - 9) * dt \quad (2)$$

The rate of photosynthesis (r_{PS}) depends on CO_2 -concentration and is modified by pH (pHM) based on a

Gaussian distribution around a pH-optimum of 7.5 with a standard deviation of 3.

$$r_{PS} = (10 + 1.2 * CO_2) * pHM \quad (3)$$

$$pHM = 1 / (3 * (2 * \pi)^{0.5}) * e^{-(pH - 7.5)^2 / 18}. \quad (4)$$

pH depends on the CO_2 -concentration. The conversion is based (i) on the formation of carbonate $[CO_2] / [H_2CO_3] = 400/1$ and (ii) on its dissociation $[H^+] \times [HCO_3^-] / [CO_2] = 4 \times 10^{-7}$, giving the following function: $[H^+] = ([CO_2] \times 4 \times 10^{-7})^{0.5}$. The constant of 3 is added to bring the pH in the model into a physiological range, and the factor 10^{-5} is introduced to bring the arbitrary units of CO_2 into the millimolar range.

$$pH = 3 - \log_{10}((CO_2 * 10^{-5} * 4 * 10^{-7})^{0.5}). \quad (5)$$

Model 2 Zeitnehmer Feedback

The underlying oscillator is essentially the same as that used in previous modeling of circadian mechanisms (Roenneberg and Merrow, 1998), but without the incorporation of temperature effects (Fig. 2). The oscillator is composed of two components (state variables), S_1 and S_2 . Their concentration at any given time (t) is defined by the rate of synthesis (P) and its rate of degradation (D).

$$S_1(t) = S_1(t - dt) + (P_1 - D_1) * dt. \quad (6)$$

The production of S_1 (P_1) is inhibited by S_2

$$P_1 = 6.5 * e^{(-0.025 * S_2)}. \quad (7)$$

The synthesis rate of S_1 directly influences its own degradation (D_1).

$$D_1 = 4 + 0.018 * S_1. \quad (8)$$

The concentration of the second component (S_2) is a direct function of the concentration of S_1 .

$$S_2(t) = S_2(t - dt) + (S_1 - D_2) * dt. \quad (9)$$

Degradation of S_2 (D_2) has a basic rate (6) and depends on a constant (15) representing the impact of an input pathway to the oscillator (note that input transduction pathways may have a "dark current," even if no external signal, that is, constant light, is provided). The

impact of the input on degradation is influenced by a feedback function (*zeitnehmer*) that depends on the concentration of S_1 .

$$D_2 = 6 + 15 * Z_{\text{eitnehmer}}. \quad (10)$$

The *zeitnehmer* function could be seen as an analogy to eyelids. If one of the outputs of the SCN closes the eyelids at a defined time of the cycle (e.g., whenever the concentration of S_1 is on the rise, see equation below), the light reaching the SCN is reduced by a factor (RF). Even under constant light, the clock generates its own light cycle. We have called these endogenously generated cycles *zeitnehmers* (time takers) (Roenneberg et al., 1998) in distinction to externally generated cycles (*zeitgebers*, or time givers). A n RF of 1 simulates no influence within this feedback loop, an RF of 0 simulates complete darkness regardless of the existing light.

$$\text{If } dS_1/dt > 0, \text{ then } Z_{\text{eitnehmer}} = 1, \\ \text{else } Z_{\text{eitnehmer}} = RF. \quad (11)$$

The initial values of S_1 and S_2 are 26 and 5, respectively.

Model 3 Influence of Metabolism

To investigate the influence of metabolism on oscillators with and without *zeitnehmer* feedback, equations 7 through 11 (indicated by the subscript m) were modified so that metabolism (M) (including temperature changes) could influence the rates within the equations.

$$P_1 = (6.5 * M) * e^{(-0.025 * S_2)}. \quad (7_m)$$

$$D_1 = (4 * M) + 0.018 * S_1. \quad (8_m)$$

$$S_2(t) = S_2(t - dt) + ((S_1 * M) - D_2) * dt. \quad (9_m)$$

$$D_2 = (6 * M) + 15 * Z_{\text{eitnehmer}} \quad (10_m)$$

$$\text{If } dS_1/dt > 0, \text{ then} \\ Z_{\text{eitnehmer}} = 1 \\ \text{else } Z_{\text{eitnehmer}} = RF - M * 1.4. \quad (11_m)$$

The influence of metabolism (M) was either a constant level (0.0 - 0.2) or randomized within defined limits (0.0 - 2.0) for each iteration (30 min).

RESULTS AND DISCUSSION

Metabolic Oscillators

Marine unicellular organisms have probably been the pioneers in evolving circadian systems. Photosynthesizing cyanobacteria, both marine (Roenneberg and Carpenter, 1993) and fresh water (Johnson et al., 1996), have regulated their metabolism on a circadian basis for perhaps millions of years. A self-sustained oscillation must have been built from cellular functions, which were initially driven by daily environmental changes, for example, photosynthesis.

The rate of photosynthesis is under circadian control in plants and algae (Sweeney, 1987) including *Gonyaulax* (Hastings et al., 1961; Samuelsson et al., 1983). Yet, light not only is a resource for circadian outputs but also affects circadian phase and period. Light reaches the *Gonyaulax* clock via two separate transduction pathways, and both apparently are under circadian control (Roenneberg and Deng, 1997; Roenneberg and Hastings, 1988; Roenneberg and Taylor, 1994). One of them is predominantly blue light sensitive and is activated only during the subjective night. The other responds both to red and blue light and is possibly associated with photosynthesis, which again is clock-controlled. The fact that photosynthesis is not only a clock output but feeds back into the circadian system was demonstrated by physiological and pharmacological manipulations of photosynthesis, which affect phase and period (Johnson and Hastings, 1989; Roenneberg, 1994).

The assimilation of nitrate is also circadian in *Gonyaulax*. Nitrate reductase, the first enzyme in this assimilation pathway, undergoes circadian oscillations both in activity and protein concentration (Ramalho et al., 1995), and we have recently found that the rate of NO_3^- uptake is also rhythmic. As with light, nitrate is not only a resource for a circadianly regulated metabolic function but acts itself as zeitgeber. The effects of light and nitrate on the *Gonyaulax* clock show strong interactions (Roenneberg and Rehman, 1996; Roenneberg and Mellow, unpublished results).

The pH of *Gonyaulax* culture medium undergoes daily changes of approximately 0.5 log units in a

Figure 1. Diagram of the photosynthesis model. For the algorithms (indicated by the equation numbers), see text.

light:dark cycle (Hastings, 1960) and free-runs with a lower amplitude (0.15 log units) in constant light (Eisensamer and Roenneberg, unpublished results). The amplitude of these pH changes corresponds to proton translocations per cell in the millimolar range. A gain, the pH rhythm is not merely an output of the *Gonyaulax* clock but appears to be a regulatory feedback loop. When the pH of the culture medium is changed experimentally, it can shift the phase of the circadian pH rhythm as well as of the bioluminescence rhythm.

These results show the strong impact of metabolic feedbacks on the daily biochemistry of *Gonyaulax*. However, can such metabolic feedbacks generate a 24-h oscillation? We addressed this question in a mathematical model (see Fig 1 and equations 1 through 5 in the Methods section), which is based on the systematic pH changes (rising during the light and falling in darkness). These pH-kinetics correlate with the rate of photosynthesis and largely reflect proton concentrations due to the dissociation of H_2CO_3 . Like most enzymatic reactions, the activity of the photosynthetic machinery depends on pH (a large body of literature exists, for example, Wong et al., 1980). This dependence is incorporated into the model as a Gaussian distribution around a pH optimum of 7.5 in equation 4. pH depends on the concentration of dissociated carbonate, which itself depends on CO_2 (equation 5). The latter is produced by mitochondrial and photorespiration, and for reasons of simplicity both are presumed to produce CO_2 at a constant rate (equation 1). Decrease of CO_2 is due to carbon fixation (CF), which

Figure 2 Diagram of the zeitnehmer model. For the algorithms (indicated by the equation numbers), see text.

depends on the rate of photosynthesis (equation 2). Finally, photosynthesis rate depends both on CO_2 -concentration and on pH (equations 3 and 4).

The chain of events connecting photosynthesis, CO_2 , and pH constitutes a metabolic feedback, and given the right parameters, the system oscillates with a circadian period (Fig. 3). The changes in pH produced by this feedback are surprisingly small (7.84-7.92). The large per cell proton oscillations experimentally measured in the culture medium reflect mechanisms of the cell that aim to keep intracellular pH in a physiological range in spite of a large proton turnover due to photosynthesis. Although the model is purely theoretical, experimental results show both changes of plastidic pH (Rappaport and Lavergne, 1991) and pH-dependencies of chloroplastic reactions (Bruce et al., 1997; Shutova et al., 1997). In addition, the circadian regulation of photosynthesis in *Gonyaulax* is mainly due to changes in photosystem II (Samuelsson et al., 1983), and pH changes alter the activity balance between the two photosystems (Braun et al., 1991).

A Self-Created, Cyclic Microenvironment

One of the hallmarks of circadian clocks is their ability to oscillate without damping in constant laboratory conditions, that is, without a natural exogenous

Figure 3 A photosynthetic oscillator due to metabolic feedback (see equations for Model I in the Methods section). CO_2 (right ordinate on the left of the graph and stippled curve), pH (ordinate on the right of the graph, dark solid curve), and photosynthesis (leftmost ordinate, gray thick curve) produce circadian oscillations. For algorithms of the photosynthesis model, see equations 1 through 5 in the Methods section. Note that the fact that photosynthesis is effectively shut off in this model during parts of the cycle does not correspond to biological reality.

temporal structure. Except for a few isolated cases (hibernators, deep sea fish), organisms never encounter constant environmental conditions. It has been argued that circadian oscillations have to be endogenously produced (and entrained to the rhythmic world) to enable anticipation and measurement of photoperiod. The endogenous nature of the circadian

Figure 4 The circadian system in *Gonyaulax* regulates behavior and metabolism, thereby producing a cyclic microenvironment even in so-called constant experimental conditions. These self-created cycles may exist in the intra- and extracellular environment of all cells including those of multicellular organisms and may be responsible for the self-sustainability of circadian rhythmicity in constant conditions.

rhythm is especially important for photoperiodic measurement because without internal reference time (i.e., when daily events are merely driven by environmental changes), systematic measurements of day length over the course of the year would be impossible. While there is no doubt about the importance of the necessity for endogenous rhythmicity, anticipation and photoperiodic measurement would also be possible with an endogenous oscillation that gradually damps in constant conditions. Since organisms are always subjected to environmental changes, there was no selection pressure for a development of long-term self-sustainability.

We argue here that long-term self-sustainability is a consequence of, rather than a prerequisite for, circadian time keeping. Circadian clocks regulate practically all aspects of cellular metabolism and, thus, create their own endogenous cyclic environment. When *Gonyaulax* cultures are kept in small culture vessels, many extracellular qualities of the medium oscillate in a circadian fashion (e.g., pH and nitrate concentration, see Fig. 4). In addition to the changes in the medium, *Gonyaulax* also systematically varies its exposure to light over the course of the circadian cycle (Roenneberg and Hastings, 1993). Thus, the algae live in their own cyclic environment even in so-called constant experimental conditions. All of these periodic factors (pH, light, or nitrate) can affect circadian qualities. Thus, feedbacks are formed between the circadian system, its outputs, the immediate environment, and finally back to the clock. The self-created cycles in the microenvironment are strong enough to synchronize

algal cultures that were out of phase before mixing, but notably only if the medium is not exchanged (Broda et al., 1985; Roenneberg and Mittag, 1996).

The oscillating medium components measured in small experimental vessels reflect intracellular changes that are most likely also present under natural conditions. They effectively create circadian signals that are independent of those created externally by the rotation of the earth (zeitgebers, or time givers) and, thus, are zeitnehmers (time takers) that also modulate the responsiveness of the circadian system to zeitgebers (Roenneberg et al., 1998).

Zeitnehmer Feedbacks Make Dampened Oscillators Self-Sustained

Although the described photosynthesis model results in self-sustained rhythmicity, such metabolic feedback loops would produce imprecise or dampened oscillations, which also are affected by nutrient concentrations, temperature, and many other effects. While zeitgebers entrain circadian systems to the 24-h rotation of the world, zeitnehmers may contribute to the robustness and the self-sustainability of circadian rhythmicity. The amount of change necessary to turn a dampened oscillation into an unabated, self-sustained rhythm is small, as can be shown by the second model (Figs. 2 and 5). The basic circadian oscillator is the same that we have used in a previous model on the role of feedback in circadian systems (Roenneberg and Merrow, 1998). It consists of two state variables that form a negative feedback (equations 6 through 11 in the Methods section). The parameters for this oscillator are chosen so that it produces rhythmicity in the circadian range but dampens over time (Fig. 5B). An output of this oscillator closes a feedback comparable to eyelids being under circadian control and, thus, modulating the amount of light reaching the circadian oscillator. The light shielded by the circadian closure of eyelids has been shown to affect the circadian period in humans (Klerman et al., 1996). When the impact of this feedback loop on the oscillator is varied systematically (analogous to the amount of light the eyelids actually shield off), the model predicts that a change of 5% is sufficient to convert a dampened into a self-sustained oscillation (Fig. 5A, C).

The oscillator in this model generates the rhythmicity, while the zeitnehmer loop supplies self-sustainability. Both elements belong intimately to the circadian clock but, on a theoretical level, they can be distinguished. Generic circadian behavior consists of

Figure 5 Feedback loops can turn a damped into a self-sustained oscillation. For algorithms of this model, see equations 6 through 11 in the Methods section.

the following qualities (Roenneberg and Mellow, 1998): (1) rhythmicity as such (independent of its frequency), (2) circadian range of period, (3) amplitude sufficiently robust to drive output rhythms, (4) self-sustainability of rhythmicity in constant conditions, (5) temperature compensation, and (6) entrainability. All of these qualities may be due to the function of different elements in the system, as shown in this model. If only one of them is impaired (e.g., by a mutation), the result is a defective circadian clock. Yet, the distinctions between oscillator (i.e., the mechanism that generates the rhythmicity) and additional feedback loops that ensure self-sustainability (see above) or temperature compensation (see below) are not pure semantics, because they help us to design experiments that result in identifying the specific function of a circadian component within the complex clock system.

Temperature Compensation Represents General Metabolic Shielding

In addition to providing self-sustainability and robustness to the circadian oscillation, zeitnehmer loops may play a role in shielding the circadian system from metabolic variations. The filamentous fungus, *Neurospora crassa*, is an excellent model system for molecular research of the circadian system (Dunlap,

1996), but it is also a successful model organism for metabolic research.

By forward and reverse genetics, a clock gene, *frequency* (*frq*), has been described in *Neurospora* (McClung et al., 1989). Its protein product and the regulation of its transcription form an autoregulating negative feedback that is essential for normal rhythmicity in spore formation (conidial banding) (Aronson et al., 1994b). Several alleles of the *frq* gene have been isolated or engineered (Aronson et al., 1994a; Dunlap, 1996; Feldman and Hoyle, 1973), including short- and long-period mutants, as well as those resulting in apparent arrhythmicity. The two null mutants *frq*⁰ and *frq*¹ are not always arrhythmic. After several days in constant darkness, growing on certain media, rhythmic conidiation can sometimes appear (Aronson et al., 1994a; Loros and Feldman, 1986). It is argued that without FRQ protein, some metabolic oscillation controls circadian conidiation. Thus, an oscillator, with circadian characteristics exists in strains that cannot produce FRQ protein (Aronson, 1994; Loros, 1986; Mellow, 1999). The fact that the wild type shows rhythmic conidiation on all media while the null mutants do so only under special nutritional conditions indicates that this mutation also affects metabolic dependencies of the circadian system in *Neurospora*. Together with this loss of "nutritional compensation," temperature compensation is partially impaired in the apparent *frq* null as well as in some rhythmic mutants (Aronson et al., 1994a). When *frq* null mutants are submitted to temperature cycles, they systematically entrain with phase angles consistent with the known circadian characteristics (Mellow et al., 1999). *frq*⁷, a robustly rhythmic long-period mutant, shows the same characteristics in temperature entrainment and is also impaired in temperature compensation.

As described above, the behavior of metabolic oscillators depends on metabolite concentrations and metabolic reactions, which depend on temperature, with a biological Q_{10} (Sweeney and Hastings, 1960). An isolated metabolic oscillator would, therefore, not be temperature compensated. Conversely, the proposed transcriptional molecular oscillators in *Drosophila* and *Neurospora* are surely not isolated from cellular metabolism. So, how are these two oscillator concepts connected? We propose that one of the functions of the transcriptional feedback loops is to regulate downstream metabolic oscillators and that they have evolved from signal transduction pathways that are themselves under circadian control, that is, form a

Figure 6 Influence of the zeitnehmer feedback on temperature compensation. nFB simulates the oscillator without a zeitnehmer loop (front plane). equation (10_m) lacks the zeitnehmer parameter [$D_2 = (6 * M) + 15$]. At 0% feedback, the zeitnehmer loop is closed but only influenced by metabolism [$D_2 = (6 * M) + 15 * (RF - M * 1.4)$; $RF = 1$]. The planes 10% to 50% represent RF -values of 0.9 to 0.5. The level of metabolic activity (influence M) is represented on the x-axis, the strength of the zeitnehmer feedback (RF) on the z-axis, and the resulting period on the y-axis.

feedback loop. Both the *Drosophila* and the *Neurospora* transcriptional feedback loops are intimately tied to light transduction and could, therefore, function as zeitnehmer, providing a feedback that contributes to a self-sustained and precise periodicity (although not accurately 24 h) as well as compensation or shielding from metabolic variations. This hypothesis predicts that mutants with defective temperature compensation are also less effectively shielded against other metabolic changes, which can easily be verified experimentally. The metabolic shielding of the circadian system is remarkable. Many substances for which a lethal dose can be established, that is, which clearly affect cellular metabolism, have absolutely no effect on circadian phase or period as long as rhythms can still be measured up to the lethal dose. Thus, interference with metabolism can result in cell death without changing circadian properties (Edmunds, 1988; Hastings, 1960).

Figure 7 Influence of the zeitnehmer feedback on short-term variations in metabolism. The allowed degree of metabolic "noise" (M) is represented on the x-axis, the strength of the zeitnehmer feedback (RF) on the z-axis (for the description of the different planes, see legend of Fig. 6), and the resulting correlation coefficient of an autocorrelation analysis on the y-axis.

Modeling Metabolic Shielding by Zeitnehmer Loops

The third model (see Materials and Methods) assesses the role of zeitnehmer feedbacks as shields from different metabolic activity levels (i.e., different constant temperatures) or from short-term metabolic variations. Temperature compensation without any influence of a zeitnehmer feedback (front panel in Fig. 6) yields a temperature-dependent circadian oscillator (comparable with frq). With moderate strength of the feedback (varied by the reduction factor, RF , see description for Model 2), temperature compensation increases with relatively weak feedback strength and decreases again at higher levels of feedback. When the reduction reaches 50% ($RF = 0.5$), temperature is compensated for as poorly as with no zeitnehmer feedback (compare rear and front panel in Fig. 6).

Zeitnehmer feedbacks not only can compensate for different overall levels or rates of metabolic activity (i.e., induced by different constant temperatures) but they can also shield the circadian system from short-term metabolic variations ("noise"). Noise was modeled by assigning random values to the activity level of metabolism (M) at each given time and varying its

allowed degree (Fig. 7). Resulting rhythms were analyzed by autocorrelation, and the correlation coefficient (r) was used as a measure for shielding (i.e., the higher r , the less impact of metabolic noise on the rhythm).

Without zeitnehmer feedback, rhythmicity is severely impaired by metabolic noise. When the feedback loop is closed but has no impact on the oscillator except for the metabolic noise itself (see legend to Fig. 6), the influence of metabolism on the robustness of rhythmicity is even greater. However, with increasing strength of the feedback, the system becomes increasingly shielded from metabolic noise (for reference, a contour of $r < 0.5$ is indicated by the stippled area).

In the zeitnehmer function (equation 11_m), the influence of metabolism is multiplied by 1.4. In modeling compensation to different overall levels of metabolic activity as well as to random metabolic noise, this factor turned out to be of great importance. The higher its value, the more the system is compensated against different overall levels (temperature compensation); the lower the value, the better it is compensated against metabolic noise.

CONCLUSIONS

In recent years, essential molecular components of the circadian system have been discovered and several aspects of their function have been described (Dunlap, 1999; Roenneberg and Meroow, 1999). In *Drosophila* and *Neurospora*, key molecular elements describe a negative feedback and have been proposed to constitute the circadian loop that generates rhythmicity in the circadian range (*frq* and *white collar* in *Neurospora*; *per*, *tim*, *clock*, *bmal* in *Drosophila*). Clearly, these components are essential for maintaining circadian oscillations with all of their known properties, but are they actually generating rhythmicity? Theoretically, the behavior of elements within a negative feedback that is under circadian control cannot be distinguished from oscillator components. In addition, experimental results indicate that a residual oscillator exists outside of the negative feedback of the *frequency* gene in *Neurospora* (Aronson et al., 1994a; Loros and Feldman, 1986; Meroow et al., 1999).

In this theoretical paper, we have addressed the possible function of feedbacks outside of the central circadian loop that generates the rhythmicity and have come to the conclusion that they may add the following properties to the system: (1) they provide

robustness to the oscillator and, thus, may be responsible for its self-sustainability; (2) they ensure that rhythmicity is in the circadian range; (3) they provide a compensation mechanism against metabolic noise and different levels of metabolic activity and, thus, may be responsible for temperature compensation; (4) they serve as zeitnehmers for external stimuli (zeitgebers) and, thus, may be contributing to the time of day specific responsiveness of the circadian system (PRC). All these putative functions make zeitnehmer loops important elements of the circadian system without necessarily being part of the mechanism that generates the rhythmicity. The transcriptional negative feedback described in insects and fungi could provide the oscillator mechanism, but they also could function as zeitnehmer loops. But what other cellular function would then provide the rhythmicity? We propose here that the generation of rhythmicity could involve elements of cellular metabolism that rhythmically change pH, redox, Ca^{2+} , energy charge, and other metabolic substrates. The negative feedback necessary for rhythmicity could be a consequence of the buildup of a cellular milieu that gradually inhibits the functions that are responsible for this buildup. A theoretical example for such a metabolic feedback is shown in the first model concerning photosynthesis.

An indication that metabolism may participate in the generation of circadian rhythmicity while nuclear reactions might contribute, as zeitnehmers, to the phasing of the rhythm and its entrainment comes from experiments with the giant alga *Acetabularia*. The rhythm of chloroplast migration continues in cells that have their nucleus removed (Schweiger et al., 1964b; Sweeney and Haxo, 1961). However, when nuclei are transplanted between cells that had been grown in opposite light/dark cycles, the nucleus determines the phase of the chloroplast rhythm (Schweiger et al., 1964a).

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