Modeling the molecular regulatory mechanism of circadian rhythms in *Drosophila*

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Summary

Thanks to genetic and biochemical advances on the molecular mechanism of circadian rhythms in *Drosophila*, theoretical models closely related to experimental observations can be considered for the regulatory mechanism of the circadian clock in this organism. Modeling is based on the autoregulatory negative feedback exerted by a complex between PER and TIM proteins on the expression of *per* and *tim* genes. The model predicts the occurrence of sustained circadian oscillations in continuous darkness. When incorporating light-induced TIM degradation, the model accounts for damping of oscillations in constant light, entrainment of the rhythm by light-dark cycles of varying period or photoperiod, and phase shifting by light pulses. The model further provides a molecular dynamical explanation for the permanent or transient suppression of circadian rhythmicity triggered in a variety of organisms by a critical pulse of light. Finally, the model shows that to produce a robust rhythm the various clock genes must be expressed at the appropriate levels since sustained oscillations only occur in a precise range of parameter values. *BioEssays* 22:84–93, 2000.

Introduction

Rhythmic phenomena occur at all levels of biological organization, with periods ranging from less than a second to years.[1,2] Among these rhythms, circadian oscillations, which occur with a period of about 24 h, play a key physiological role in the adaptation of living organisms to their periodically varying environment. Experimental advances during the last decade have contributed to the unravelling of the molecular bases of circadian rhythms in a number of organisms, the best studied so far being *Drosophila*.[3–6] Significant progress has also been made with the mechanism of circadian rhythms in *Neurospora*,[7,8] cyanobacteria,[9,10] plants[11,12] and mammals.[13–15]

Oscillatory behavior often originates at the cellular level from regulatory feedback loops which involve many parameters and interacting variables. Relying on sheer intuition to predict the dynamics of such complex regulatory systems rapidly meets with limitations. Analyzing the origin of oscillations has much to gain therefore from theoretical models closely related to experimental observations. Some of the roles and advantages of theoretical models in biology are listed in Table 1. Considerations of the use of theoretical models apply to the study of biological processes in general, but pertain particularly to biological rhythms which only occur in precise conditions. Determining these conditions is a primary goal of a modeling approach.

Models have been applied primarily to ultradian biochemical oscillations, characterized by periods ranging from seconds to minutes.[1,2] Theoretical models for circadian rhythms were at first borrowed from the physical literature, as exemplified by the use of the van der Pol oscillator for modeling properties of circadian oscillations.[16] This line of research is still pursued to study, for example, the effect of light on the human circadian system.[17] Besides these abstract models, a complementary approach rests on the study of models more directly related to the biochemical regulatory processes that underlie circadian rhythms. In nearly all cases investigated so far, it appears that a central role in the mechanism of circadian rhythmicity is played by autoregulatory loops of negative feedback on gene expression.[18,19] Given the increasing availability of experimental data, detailed theoretical models can now be considered for circadian rhythms. Such models based on transcriptional regulation have been proposed for the two best-characterized examples, namely the circadian oscillations of the products of the *per* and *tim* genes in *Drosophila*[20–23] and of the product of the *frq* gene in *Neurospora*.[22–24]

In the present article we examine a model for circadian oscillations based on the experimental observations available about the molecular mechanisms of circadian rhythms in *Drosophila*. Incorporating the effect of light on the circadian mechanism allows the comparison of theoretical predictions with experimental data in regard to several properties, including oscillations in continuous darkness or light, entrainment by

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TABLE 1. Some Roles and Advantages of Theoretical Models in Biology

- Provide a unified theoretical framework accounting for available experimental observations, that corroborates or not experimental conclusions.
- Conceptualization leads to clarification of hypotheses.
- Analyze complex situations involving multiple, coupled variables, for which it becomes impossible to rely only on sheer intuition.
- Models show that certain types of behavior only occur in precise conditions, in a domain bounded by critical parameter values, in contrast to what may be predicted by merely verbal descriptions.
- Determine the qualitative and quantitative effects of each parameter and identify key parameters.
- Rapid exploration of different mechanisms and of large ranges of conditions.
- Possibility to ask questions which may be inaccessible to experiments or hard to address experimentally.
- Testable predictions: suggestion of experiments, which will either validate the model or call for its modification.
- Optimal situation: provide counterintuitive explanations or surprising predictions.
- Mathematical structure underlines link with similar phenomena in other contexts.

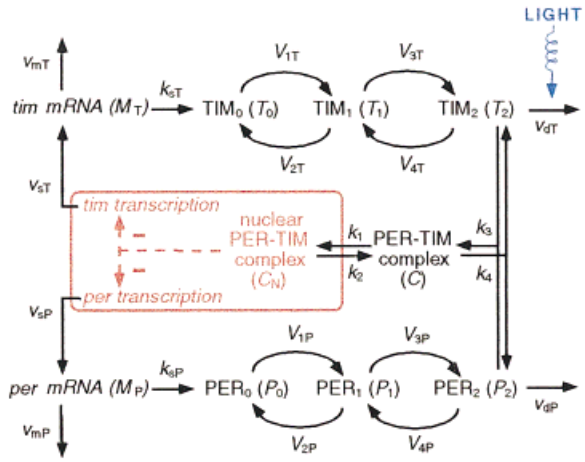

Figure 1. Scheme of the model for circadian oscillations in *Drosophila*.[21] The model is based on the negative regulation exerted by the PER–TIM protein complex on the expression of the *per* and *tim* genes; light controls the rhythm by enhancing the rate of TIM degradation, v_{dT}. The model incorporates gene transcription in the nucleus, accumulation of the corresponding mRNAs and protein synthesis in the cytosol, protein and mRNA degradation, phosphorylation-dephosphorylation of PER and TIM, protein transport into and out of the nucleus, formation of a PER-TIM complex and regulation of gene expression by the nuclear form of this complex. Nuclear processes are shown in red and the effect of light is indicated by the blue arrow.

light-dark cycles, and phase shifting by light pulses. We also use the model to address the transient or permanent suppression of circadian rhythmicity by critical pulses of light. Finally, the model shows that sustained oscillations only occur in a restricted domain of parameter values. Thus, the transcription of the various clock genes does not necessarily lead to circadian rhythmicity. The production of a robust circadian rhythm requires that these genes be expressed at the appropriate levels ensuring that the genetic control system operates within a domain of sustained oscillations.

Model for circadian rhythms in *Drosophila*

The model for circadian oscillations in *Drosophila* is represented schematically in Figure 1. The *per* and *tim* genes are transcribed in the nucleus before the mRNAs are transported into the cytosol where they are translated. The PER and TIM proteins are phosphorylated at multiple sites[25,26] and form a complex that enters the nucleus.[26–29] Oscillations are also obtained when the complex is assumed to involve the non-phosphorylated forms of PER and TIM. Through interaction with the products of the activator genes *clock*[30,31] and *cyc*[32] (not shown in the Figure) the PER-TIM complex inhibits expression of *per* and *tim* genes. The model for circadian rhythms in *Drosophila* incorporating the formation of the PER–TIM complex is described by a set of ten kinetic equations.[21] Three additional equations are needed to describe the dynamics in the presence of the *clock* and *cyc* genes (J.-C. Leloup and A. Goldbeter, unpublished data). Light controls the rhythm by triggering the destruction of the TIM protein[26–29] through a ubiquitin-proteasome mechanism that requires TIM phosphorylation.[33]

Circadian oscillations in continuous darkness

Circadian rhythms are necessarily endogenous. Accordingly, the model schematized in Figure 1 predicts the occurrence of sustained oscillations in constant darkness (DD), as observed experimentally. Shown in the top panel of Figure 2 are the oscillations in total PER protein (P_t), *per* mRNA (M_P), and nuclear PER–TIM complex (C_N) observed in conditions corresponding to DD; such conditions are achieved in the *Drosophila* model by holding the parameter v_{dT}, which measures the maximum rate of TIM degradation, at a constant low value (see Fig. 1). Although the environmental conditions remain constant, the PER–TIM control system generates autonomous oscillations with a period close to 24 h for the set of parameter values considered.

As expected from mechanisms in which a protein represses the transcription of its gene,[18] the model for *Drosophila* predicts that the peak in mRNA levels precedes the peak in protein by several hours. Moreover, the peak in total

PER and TIM precedes the peak in nuclear PER–TIM complex (Fig. 2, top panel).

Circadian clocks as limit cycle oscillators

When plotting the time course of the system as a function of the concentrations of two of the biochemical variables in constant darkness, sustained oscillations (Fig. 2, top panel) take the form of a closed curve (Fig. 3). For a given set of conditions (i.e. of parameter values), this closed curve is obtained regardless of initial conditions and is generally unique; hence the name *limit cycle* given to this type of trajectory associated with periodic behavior. Because perturbations do not change their period or amplitude in the long run, limit cycle oscillations represent a particularly stable mode of periodic behavior. Such stability holds with the robust nature of circadian clocks which have to maintain their amplitude and period in a changing environment while retaining the capability of being phase shifted by light or temperature.

In Figure 3, the trajectory toward limit cycle is shown as a projection onto the plane formed by the concentrations of *per* mRNA and total PER protein. On the curve, the direction of movement (arrow) is such that the maximum in mRNA precedes the maximum in protein as observed in the experiments.[18] This holds with the fact that the rising protein level brings about the fall in transcription.

Control of circadian rhythms by light

Light triggers degradation of the TIM protein in *Drosophila*.[26–29,33] Building a periodic variation of the light-controlled parameter into the model of the *Drosophila* circadian clock allows us to simulate the entrainment of circadian

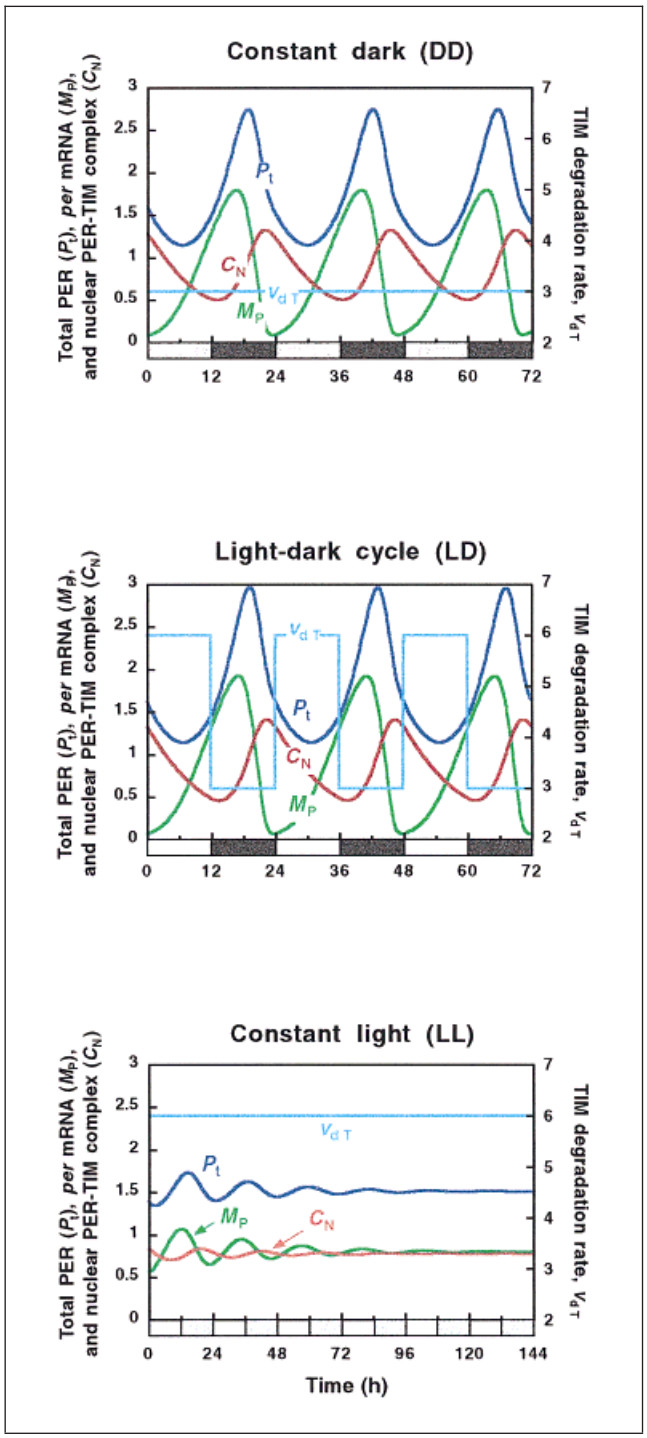

Figure 2. Oscillations in continuous darkness, entrainment by LD cycles, and behavior in continuous light in the model for circadian rhythms in *Drosophila*. The top panel corresponds to continuous darkness, the middle panel corresponds to entrainment by a light-dark cycle of 24 h period (12:12 LD) and the bottom panel corresponds to continuous light. The LD cycle is symbolized by the alternation of white and black bars; continuous darkness is symbolized by the alternation of gray and black bars. The curves are obtained by numerical integration of the ten kinetic equations governing the dynamics of the extended model schematized in Figure 1; these equations are listed as eqs. (1a)-(1j) in ref. 21. Shown is the temporal variation in *per* mRNA (M_P) and in the total amount of PER protein (P_t), together with the variation in nuclear PER-TIM complex (C_N). Parameter values are: $n = 4$, $v_{sP} = 1.1$ nMh^{-1}, $v_{sT} = 1$ nMh^{-1}, $v_{mP} = 1.0$ nMh^{-1}, $v_{mT} = 0.7$ nMh^{-1}, $v_{dP} = 2.2$ nMh^{-1}, $k_{sP} = k_{sT} = 0.9$ h^{-1}, $k_1 = 0.8$ h^{-1}, $k_2 = 0.2$ h^{-1}, $k_3 = 1.2$ nM^{-1}h^{-1}, $k_4 = 0.6$ h^{-1}, $K_{mP} = K_{mT} = 0.2$ nM, $K_{IP} = K_{IT} = 1$ nM, $K_{dP} = K_{dT} = 0.2$ nM, $K_{1P} = K_{1T} = K_{2P} = K_{2T} = K_{3P} = K_{3T} = K_{4P} = K_{4T} = 2$ nM, $V_{1P} = V_{1T} = 8$ nMh^{-1}, $V_{2P} = V_{2T} = 1$ nMh^{-1}, $V_{3P} = V_{3T} = 8$ nMh^{-1}, $V_{4P} = V_{4T} = 1$ nMh^{-1}, $k_d = k_{dC} = k_{dN} = 0.01$ nMh^{-1}. v_{dT} (in nMh^{-1}) remains constant and equal to 3 in the top panel and to 6 in the bottom panel, and is equal to 3 and 6 in the middle panel during the dark and light phases, respectively. The concentration scale in each graph is expressed, tentatively, in nM. Given that quantitative experimental data are still lacking, the above parameter values have been selected arbitrarily, though in a physiological range, so as to yield a free-running period close to 24 h.

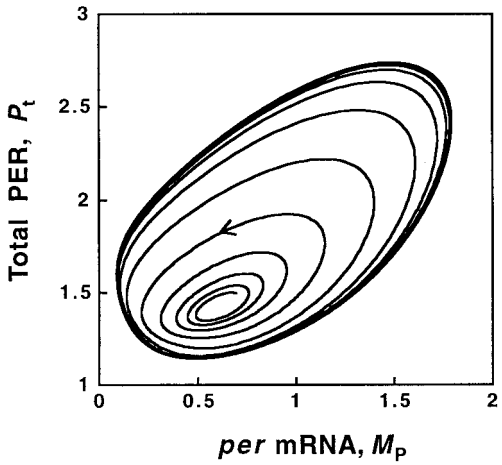

Figure 3. Evolution toward a limit cycle corresponding to sustained oscillations in the model for circadian oscillations in *Drosophila*. The oscillations are those observed in conditions of continuous darkness in the top panel of Figure 2. The limit cycle is reached here from initial conditions located near the unstable steady state. The arrow indicates the direction of movement along the limit cycle.

oscillations by light-dark (LD) cycles, as shown in Figure 2 (middle panel). In such conditions, the maximum TIM degradation rate v_{dT} varies in a square-wave manner as it increases up to a higher value during each light phase. As the duration of both the light and dark phases is equal to 12 h in the case considered (this particular light-dark cycle is denoted by 12:12 LD), the system is entrained precisely to the 24 h external periodicity. In constant darkness, for the parameter values considered, the model predicts a free-running period close to 24 h, as observed experimentally.[3]

The effect of continuous light (LL) is simulated by holding v_{dT} at a constant high value. As observed experimentally,[34] the oscillations in the model are readily damped in LL by increasing v_{dT} to a sufficient, constant value (Fig. 2, bottom panel).

Sustained versus damped oscillations

The fact that all the parts of a clock mechanism are present does not ensure that the mechanism necessarily produces a rhythm. Indeed, sustained oscillations of the limit cycle type only occur in appropriate conditions, beyond critical values or, more often, in a window bounded by two critical values of some control parameters. The latter situation is schematized in Figure 4A, in the case where a control parameter (denoted by λ) is affected by light. At low values of λ, the system reaches a stable steady state corresponding to some constant concentrations of the state variables, only one of which

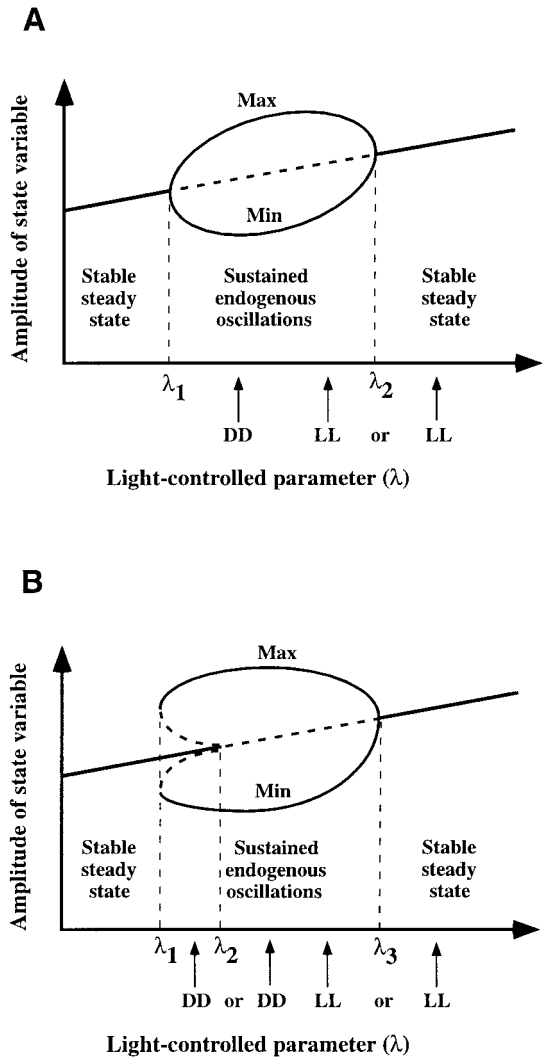

Figure 4. Schematic bifurcation diagrams as a function of a light-controlled parameter λ. Each diagram represents as a function of the control parameter λ the steady-state value of a state variable, which is either stable (solid line) or unstable (dashed line), and the maximum and minimum of this variable in the course of sustained oscillations. **A**: a stable limit cycle exists between λ_1 and λ_2; the steady state in this region is unstable. **B**: a stable limit cycle coexists with a stable steady state between λ_1 and λ_2 (the dashed line that separates them refers to an unstable limit cycle); the situation between λ_2 and λ_3 is the same as in panel A. The arrows at the bottom of each diagram exemplify conditions (values of λ) corresponding to continuous darkness (DD) or light (LL).

is represented. As λ increases, a bifurcation occurs when a critical value λ_1 is passed: the steady state becomes unstable and sustained oscillations occur, the amplitude of which increases and passes through a maximum as the value of

the control parameter rises further. When a second critical value λ_2 is exceeded (provided that such a critical value exists), sustained oscillations disappear and the system again evolves toward a stable steady state.

To relate the results shown in Figure 2 to the scheme of Figure 4A, sustained oscillations observed in constant darkness should correspond to a value of the light-controlled parameter λ located in the domain of sustained autonomous oscillations, i.e. between λ_1 and λ_2. If oscillations are damped in constant light, the value of λ in these conditions should increase to a value higher than the values required to support sustained oscillations, i.e. above λ_2. Denoting the values of the light-controlled parameter in constant darkness and constant light by λ_{DD} and λ_{LL}, the following relations would then hold: $\lambda_1 < \lambda_{DD} < \lambda_2 < \lambda_{LL}$. If sustained oscillations were to persist in continuous light, these relations would be replaced by: $\lambda_1 < \lambda_{DD} < \lambda_{LL} < \lambda_2$.

Another situation can be encountered, however, as schematized in Figure 4B. In this case, in a domain bounded by two critical values λ_1 and λ_2, a stable steady state coexists with a stable limit cycle, from which it is separated by an unstable cycle. This situation is known as *hard excitation*: a finite perturbation is needed to leave the stable steady state and to evolve to the stable limit cycle (such a situation was already envisaged when circadian rhythms were first related to limit cycle behavior[35]). In contrast, an infinitesimal perturbation is needed to evolve to the limit cycle when the steady state is unstable, in the interval between λ_2 and λ_3. While the cases of oscillations in DD or LL can be discussed as for the situation in Figure 4A, we will explore below the dynamical consequences of the situation shown in Figure 4B.

The role of the light-controlled parameter λ in the *Drosophila* model is played by the rate of TIM degradation, v_{dT}. The analysis indicates that the domain of sustained oscillations is bounded by two critical values of v_{dT}. Below the lower critical value, the concentration of the PER-TIM complex is so high that it prevents circadian cycling. Above the higher critical value of v_{dT}, the PER-TIM complex is degraded so rapidly that it cannot reach the level required for effective repression of the *per* and *tim* genes. The fact that the rhythm is sustained in DD but damped in LL suggests that the value of v_{dT} in DD and LL lies, within and outside the range for sustained oscillations, respectively. Depending on other parameter values, hard excitation may or may not be observed as a function of v_{dT}.

Phase shifts induced by light pulses

The induction of phase shifts by light pulses represents one of the most conspicuous properties of circadian rhythms. Since we have incorporated the effect of light into the model for *Drosophila* circadian rhythms, we can use this model to determine the response to light pulses as a function of the phase at which the system is perturbed. We can use these results to construct phase response curves (PRCs) to compare with those determined experimentally. The question remains as to the actual duration and amplitude of the biochemical changes brought about by the pulses of light applied in these experiments. Even if the pulses are brief, the consequent parameter changes can be long-lasting: a 1 min pulse of light can indeed turn on a gene and thereby elicit the synthesis of an enzyme that may remain active for hours. By allowing the construction of families of PRCs for different durations and magnitudes of the effects caused by a light pulse, the models can be used to predict the amplitude-duration pairs that yield good agreement with the experimental curves.

The best fit with experimental PRCs determined for the wild type and short period (*per*^{s}) mutant in *Drosophila* is obtained[21] when assuming that the effect of a 1 min light pulse is to multiply the maximum degradation rate of the TIM protein by a factor of 2 over 3 h, although such a combination is by no means unique. Shown in the top panel of Figure 5 are the unperturbed oscillations (blue curve) in the fully phosphorylated, degradable form of TIM (T_2), as well as the delay and phase advance resulting from the application of such a perturbation early (red curve) and late (green curve) in the rising phase of this TIM form. Thus, as observed experimentally,[26–29] the increase in TIM degradation triggered by light causes a phase delay during the rising phase of TIM (and PER), and a phase advance when TIM is close to its maximum value. The absence of phase shift corresponds to the times at which TIM is near its minimum; any increase in TIM degradation should indeed have negligible effects at such phases of the oscillations.

Shown in Figures 5A and 5B are the theoretical PRCs determined for wild type *Drosophila* and the *per*^{s} mutant. The corresponding unperturbed oscillations in TIM and *per* mRNA are shown in Figures 5C and 5D, respectively; phase 0 is set so that *per* mRNA reaches its minimum after 12 h, close to the beginning of the subjective day (see Fig. 2, top panel). The theoretical PRCs (solid lines) compare well with the experimental results[36] (dots). The main difference between the PRCs for the wild type and *per*^{s} flies is that the dead zone, in which light pulses fail to elicit any significant phase shift, is much smaller for the mutant. As shown by a comparison between Figures 5C and 5D, the model suggests that this difference arises because the TIM protein spends less time near its minimum in the *per*^{s} mutant. The enhanced degradation rate of the nuclear PER-TIM complex in the mutant[37] would lead to a reduced depression of mRNA levels and, hence, to a precocious rise in TIM.

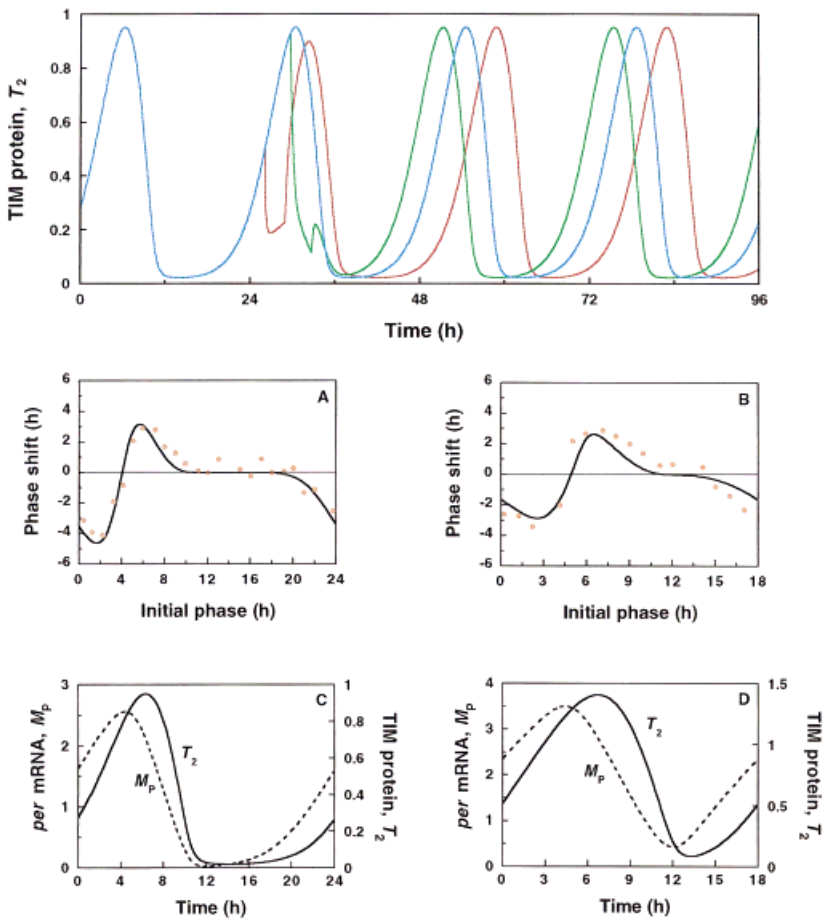

Figure 5. Phase shifts induced by light pulses in the model for the *Drosophila* circadian clock. The top panel shows the unperturbed oscillations of the fully phosphorylated form, T_2, of TIM (blue curve), as well as the phase shifts induced by pulses applied at two different phases of the oscillations, yielding a phase delay (red curve) or a phase advance (green curve). The lower panels show the phase response curves (PRCs) obtained experimentally (dots) and theoretically (solid lines) for the wild type (**A**) and the *per*^{s} mutant (**B**) in *Drosophila*, following perturbation by a light pulse. The PRC yields the phase shift (positive for phase advances, negative for phase delays) as a function of the phase of perturbation. The experimental points, obtained by Orr and Konopka using a 1 min light pulse, are redrawn from Figure 2 of Hall and Rosbash.[36] The theoretical PRCs have been obtained by integrating the model equations (eqs (1a)-(1j) in Ref. 21), starting with initial conditions corresponding to the particular phase of the unperturbed oscillations in (**C**) and (**D**), after multiplying by a factor of 2 during 3 h parameter v_{dT} which measures the maximum rate of degradation of the fully phosphorylated form of TIM. (C) and (D) show the unperturbed oscillations in the TIM protein (T_2) and the *per* mRNA (M_P) for the cases of the wild type and *per*^{s} mutant, respectively; in each case phase zero is chosen as in the experiments[36] so that the minimum in *per* mRNA occurs after 12 h. Phase zero thus corresponds to the beginning of the subjective night. Parameters in (C) and for the solid line in (A) (corresponding to the wild type) are as in Figure 2 (top panel), with $k_1 = 0.6\ h^{-1}$, $v_{sP} = 1\ nMh^{-1}$, $v_{mP} = 0.7\ nMh^{-1}$, $v_{dP} = v_{dT} = 2\ nMh^{-1}$. For (D) and the solid line in (B) (corresponding to the case of the *per*^{s} mutant), the value of k_{dN} measuring the degradation of the nuclear PER-TIM complex has been increased from 0.01 h^{-1} up to the value of 0.45 h^{-1} (see also Fig. 3A in Ref. 21). The value of the free running period in (C) and (D) is equal to 24.135 h and 18.025 h, respectively.

Suppression of circadian rhythmicity by critical light pulses

The possibility of suppressing circadian rhythms by critical light pulses or other perturbations has long been considered. Winfree has suggested that such critical perturbations applied at the appropriate phase of a limit cycle should stop the clock, at least transiently, if the perturbation brings the oscillator back into the vicinity of the steady state, commonly referred to in this context as the *singularity*.[1,38] The model considered here allows us to address this issue explicitly, given that the effect of light in this model is built into the molecular mechanism of circadian oscillations.

The two situations schematized in Figures 4A and 4B have distinct dynamical consequences regarding the possibility of suppressing circadian rhythmicity by light pulses. In the situation illustrated in Figure 4B when the control parameter λ lies between the two critical values λ_1 and λ_2, a stable steady state coexists with a stable limit cycle. An unstable limit cycle separates the basins of attraction of these two stable regimes. Such a situation, observed in the model for circadian rhythms in *Drosophila*,[22] is schematized in the upper left panel in Figure 6. The arrow from the stable limit cycle (solid curve) symbolizes the effect of a light pulse that brings the system across the unstable limit cycle (dashed curve) into the basin of attraction of the stable steady state. The consequence of such a light pulse applied at the appropriate phase with the appropriate duration and magnitude is illustrated in the left bottom panel of Figure

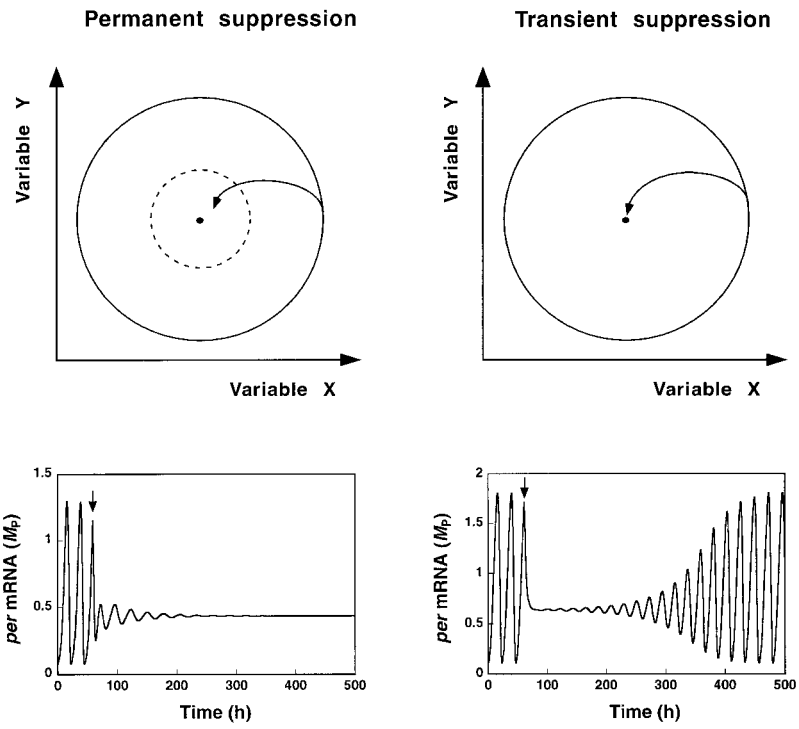

Figure 6. Permanent or transient suppression of circadian rhythmicity by light pulses in the *Drosophila* clock model. The **upper left panel** refers schematically to the coexistence between a stable limit cycle (solid curve) and a stable steady state (dot) which are separated by an unstable limit cycle (dashed line). The **upper right panel** portrays the situation of a stable limit cycle surrounding an unstable steady state. The (stable or unstable) steady state is often referred to as *singularity*. The curves in the bottom panels have been obtained by numerical integration of the kinetic equations governing the evolution of the *Drosophila* circadian clock model.[21] **Lower left panel**: permanent suppression of the circadian rhythm. Parameter v_{dT} is increased, at the time indicated by the vertical arrow, during 2 h from the basal value of 1.3 nMh^{-1} up to 4.0 nMh^{-1}. **Lower right panel:** transient suppression of the circadian rhythm in the *Drosophila* clock model. At the time indicated by the arrow, parameter v_{dT} is increased during 3.8 h from the basal value of 3.5 nMh^{-1} up to 6.7 nMh^{-1}. The basal values v_{dT} = 1.3 nMh^{-1} and 3.5 nMh^{-1} correspond to the situations depicted in the upper left and right panels, respectively. Other parameter values are as in Figure 2.

6. The light pulse has suppressed the rhythm permanently.

The analysis of the *Drosophila* model indicates that the phases at which the light pulse permanently suppresses the rhythm correspond roughly to the portion of the limit cycle associated with the rise in TIM. In the duration-amplitude space, the patterns of pulses that succeed in suppressing the rhythm change as a function of the phase, as the system goes from the minimum to the maximum in TIM (J.-C. Leloup and A. Goldbeter, unpublished data).

In the more common situation, in which a stable limit cycle does not coexist with a stable steady state (see Fig. 4A, and Fig. 4B in the interval between λ_2 and λ_3), the effect of a light pulse is different. As illustrated in the upper right panel of Figure 6, a light pulse applied at the appropriate phase with the appropriate duration and magnitude can bring the system into the close vicinity of the steady state, but because the latter is unstable the system will eventually return to the limit cycle, possibly after skipping a number of peaks, as illustrated for a higher value of v_{dT} in the *Drosophila* model in the bottom right panel of Figure 6. In such conditions, suppression of the circadian rhythm by the light pulse is only transient. As will be discussed below, both transient and permanent suppression of circadian rhythms by single, critical perturbations have been observed experimentally.

Conclusions

It has long been suggested that that circadian rhythms are produced by limit cycle oscillators and this concept was first investigated by means of abstract mathematical models or models derived from the physical sciences.[1,16,17,35,39] In recent years, many of the genes involved in circadian clock control have been identified in various organisms, initially in *Drosophila*[3–6] and *Neurospora*,[7,8] and now including cyanobacteria,[9,10] plants[11,12] and mammals.[13–15] With the rapid experimental advances concerning the underlying regulatory mechanisms, more detailed theoretical models can be considered for the origin of circadian behavior. To date models have been studied for the best characterized systems, namely *Drosophila*[20–23] and *Neurospora*.[22–24] Detailed models will undoubtfully be considered for other organisms as their clock control mechanisms are clarified further at the molecular level.

Although important differences may exist between organisms as to the precise role of light in the control of genes involved in the circadian oscillatory mechanism, some unifying principles are emerging from experimental studies and

theoretical models. Recurrent is the observation that, coupled to a positive process that drives the genetic regulatory network, negative autoregulatory feedback loops underlie circadian oscillations in a variety of organisms.[8] In *Drosophila*, a complex between the PER and TIM proteins exerts indirect negative feedback on the expression of the *per* and *tim* genes (see Fig. 1). In *Neurospora,* the protein FRQ exerts a negative feedback on the expression of its gene *frq*.[8,19] Available, albeit preliminary, data suggest that negative autoregulation of transcription might similarly underlie circadian rhythmicity in cyanobacteria, plants and mammals.[10,12,40]

The analysis of theoretical models shows that this type of negative feedback regulation can readily give rise to sustained oscillations of the limit cycle type (see Fig. 3). The model described here for circadian rhythms in *Drosophila* can be viewed as an extension of a general three-variable model proposed by Goodwin[41] soon after the principles of genetic regulation were established. Anticipating many of the subsequent experimental findings on circadian clock mechanisms, this author suggested that negative feedback on gene expression could lead to oscillations in both protein and mRNA levels. Goodwin's model was recently used to study the phase shifting of circadian rhythms by inhibitors of protein synthesis in *Neurospora*.[24] Because of its simplicity, the degree of cooperativity of repression needed to produce limit cycle oscillations in this model is very large.[42,43] Incorporating saturable kinetics for mRNA and protein degradation, as well as phosphorylation of the proteins PER and TIM in *Drosophila*, limit cycles can be obtained for smaller degrees of cooperativity in repression and even in the absence of cooperativity altogether.[21] Besides the theoretical studies already mentioned, other models based on negative control of gene expression have been proposed, which also lead to circadian limit cycle behavior. In contrast to the model discussed above, these models incorporate either discontinuous evolution equations[44] or time delays.[45,46]

The model shown in Figure 1 accounts for the occurrence of sustained oscillations in continuous darkness, for the damping of oscillations in constant light, as well as for the entrainment of the oscillations by LD cycles when incorporating the effect of light on TIM degradation in *Drosophila* (Fig. 2). The model further accounts for the phase locking of the oscillations observed in 24 h LD cycles of varying photoperiod.[34] As shown in Figure 5, the theoretical phase response curves for *Drosophila* match those obtained in the wild type and per^s mutant in response to light pulses. The model also provides an explanation for the reduction of the dead zone in which no phase shift occurs in the short period mutant and allows us to assess the effect of protein phosphorylation and PER-TIM dimerization on the oscillatory mechanism.[21]

Another use for theoretical models is to shed light on the conditions in which light pulses suppress circadian rhythmic behavior. The possibility that critical pulses of light achieve such an effect by bringing the oscillatory system back to the singularity has long been explored both experimentally and theoretically by Winfree.[1,38] As shown in Figure 6, we may distinguish between two different cases, depending on whether the stable limit cycle coexists with a stable steady state or not. In the former case, a single light pulse applied during the rising phase of TIM and inducing TIM degradation with the appropriate duration and magnitude can permanently suppress the rhythm (Fig. 6, left column). In the second situation, the rhythm is recovered after skipping a (variable) number of peaks (Fig. 6, right column). This model, based on the molecular mechanism of circadian rhythms in *Drosophila*, is the first to describe explicitly the permanent suppression of circadian rhythmicity by a single light pulse as observed experimentally. Such a suppression in which a stable steady state and a stable limit cycle coexist was previously analyzed in circadian oscillator models of the van der Pol type.[47,48] The phenomenon has, in fact, been observed in the circadian rhythm of pupal eclosion in *Drosophila pseudoobscura*.[38] It has also been reported for circadian rhythms in mammals including hamster,[49] chipmunk,[50] and humans,[51] as well as for plants such as *Kalanchoe*.[47] Ultradian illustrations of an analogous phenomenon have been reported for squid axon membranes[52] and cardiac tissue[53] in which repetitive firing was prevented by a brief depolarizing or hyperpolarizing current pulse, respectively.

The *Drosophila* model also accounts for the observation that circadian oscillations may persist in the absence of negative regulation of *per* gene expression.[54] A similar conclusion holds if the *tim* gene, rather than *per*, escapes regulation, but the model predicts that no oscillations should occur if the two genes cease to be regulated by PER and TIM. In addition to transcriptional feedback, experimental evidence points to a role for post-transcriptional regulation in the mechanism of circadian oscillations in *Drosophila*.[55,56] Incorporating the possible control of *per* mRNA degradation by the PER-TIM complex into the model indicates that such a regulation may, already on its own, give rise to sustained oscillations. When added to transcriptional control of *per* and *tim* expression, regulation of mRNA half-life by the PER-TIM complex does not markedly influence the oscillations produced by the transcriptional feedback loop.

Another conspicuous property of circadian rhythms is the relative independence of their period with respect to temperature.[57] Theoretical studies indicate that such a *temperature compensation* property arises from antagonistic effects of temperature on the various biochemical processes that make up the molecular mechanism of the circadian clock.[58,59] Thus, in the *Drosophila* model, some rate con-

stants will increase while others will decrease the period as temperature rises. If these effects roughly balance each other, temperature compensation will ensue. Such a balance is presumably lost in the *per*^{s} and *per*^{l} mutants, for which the period respectively decreases and increases as temperature rises.[60]

The model proposed for circadian rhythms in *Neurospora*[22,23] shares many features with that proposed for the *Drosophila* circadian clock. The *Neurospora* model is based on the negative autoregulatory feedback exerted by the protein FRQ on the transcription of its gene *frq*.[19] Recent experiments on FRQ-deficient mutants indicate that a second oscillator, entrainable by temperature and also related to the conidiation rhythm, may be present in *Neurospora* in addition to the light-controlled oscillator involving FRQ.[61,62] From a theoretical point of view, it will be interesting to incorporate such a second oscillator into the model for circadian rhythmicity, as details become available on its molecular mechanism and function. A somewhat abstract model has been proposed in which a second oscillator would feed back on the input to a first oscillator which, in the case of *Neurospora*, would be controlled by FRQ.[63]

The present results bear on the origin of circadian rhythms in other organisms, including cyanobacteria, plants and mammals. The majority of mammalian circadian clock genes identified so far are homologous to those of *Drosophila*.[64] The effect of light on the mammalian clock is closer, however, to that observed in *Neurospora*, since light induces gene expression rather than protein degradation in mammals.[65]

Finally models indicate that the mere presence of gene products that compose the clock mechanism does not necessarily ensure the rhythmic function of this mechanism. Indeed, as schematized in Figure 4, sustained oscillations of the limit cycle type only occur in a well defined domain in parameter space; outside this domain, oscillations damp out as the system evolves toward a stable steady state. For a certain tissue or cell type to function autonomously as a circadian oscillator therefore depends not only on the transcription of the various clock genes, but also on the precise levels at which these genes are expressed.

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