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Discrete Delay Model for the Mammalian Circadian Clock

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Delay model · Mammals · Circadian rhythms · Chaos

Abstract
A three-variable discrete delay model is proposed for the circadian rhythm of the mammals with BMAL1, PER-CRY complex and REV-ERBα protein concentrations as the dynamical variables. The delay model is phenomenological in nature rather than the precise description of all the underlying complex processes. The goal of this paper is to study the effects of delay in the circadian rhythms of mammals that appears in both the positive and negative feedback loops of the model. The delay model exhibits 24-hour limit cycle oscillations, entrainment to light-dark (LD) cycles and phase response curves. The model is also found to exhibit quasiperiodic and chaotic oscillations under LD cycles when delay is varied. These are linked to non-24-hour sleep-wake syndrome and cancer incidence. The mutations in *Bmal1*$^{-/-}$, *Per*^{Brdm1}, *Rev-Erb*$\alpha^{-/-}$ are explained in terms of delay, whereas the double mutations *Per*^{Brdm1}/*Cry*2$^{-/-}$ and *Cry*1$^{-/-}$/*Cry*2$^{-/-}$ are explained in terms of the strength of delayed positive and negative regulations. The delay model in essence captures the core mechanism of the mammalian circadian rhythms with a smaller number of variables and parameters.

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Synopsis

Day after day for many years, our internal clocks wake us around sunrise and put us to sleep at night. It all seems so natural and works so smoothly that most of us scarcely wonder about the delicate nonlinear biochemistry that is involved, or the impressive flexibility of its dynamics. The human body's natural rhythm is easily entrained by the 24-hour cycle of light and dark in our earthly environment. Our bodily rhythm adjusts itself naturally to any shift in the phase of this light-dark cycle, if we take a long flight to another time zone. Yet such changes also entail a cost – the inevitable malaise of 'jet lag' before your chemistry can re-adjust.

Scientific studies of circadian rhythms – which we share with organisms ranging from elephants to crickets – date back to the 18th century. A number of studies have shown that circadian-like dynamics can be reproduced, with at least qualitative accuracy, in nonlinear systems of ordinary differential equations representing the interactions of various genes and proteins. But many mysteries remain. Even in simple 'model' organisms, the underlying biochemical networks are exceedingly complex. Moreover, as Sriram and colleagues point out in this paper, most mathematical investigations have neglected one important biological feature – time delays. Biological regulation typically takes place through feedback, as the product of one reaction can serve to enhance or suppress another reaction. This is certainly true of circadian circuits, but, as the authors point out, for the circadian rhythms of model organisms such as *Drosophila* and *Neurospora*, and for mammals, 'there is always time lag associated with biosynthesis and transport of regulatory protein to reach the site of action and the time lag varies approximately between 8–12 h'. Hence, models of circadian rhythms should not really focus on ordinary differential equations, but on

Introduction

A circadian rhythm is an oscillation with a period of approximately 24 h, which exhibits entrainment to environmental light-dark (LD) cycles and shifting of phase by light stimulation. Even though many theoretical models with ordinary differential equation (ODE) have been proposed based on the biochemical mechanisms for circadian rhythms [1–4], relatively few studies have been carried out with delay differential equations. Delayed feedbacks are common and occur naturally in many biological systems and, in particular, the regulatory networks of circadian rhythms. It has been found that in circadian rhythms of Drosophila, Neurospora, and mammalian systems of the wild type, there is always a time lag associated with biosynthesis and transport of regulatory protein to reach the site of action and the time lag varies approximately between 8 and 12 h.

Recently, there has been a spurt of interest in employing delay differential equations for the study of circadian rhythms [5–9]. A model based on delay differential equations has the advantage of not having to specify all the processes explicitly and their effect on the dynamics of the system can be lumped in the form of delay. This feature is interesting because most of the experimental data for the system of interest are still lacking, and some of the processes that are not well understood can be lumped in the form of delay and thus reduce the number of effective variables and parameters to be fixed. Further, delay differential equation also releases the phase-space constraint of the system rendering the system infinite dimensional [10]. The disadvantage is that the nonlinear delay differential equations are notoriously difficult to solve and the usual phase plane and geometrical methods cannot be carried out for the system with delay.

In this paper, we propose a delay model for the circadian rhythm of the mammals with three dynamical variables, with delay in all the variables. The goal is not to explain experimental details exhaustively or present any new evidence to look for some hitherto unexplored aspects, but to study the effect of delays, extracted from the experiments, on the dynamics of the system with a smaller number of variables and parameters. This is because the molecular mechanism of mammalian circadian rhythms at the cellular level is too complex and the experimentation is still underway. It is not possible to obtain a complete picture of the system. Thus only the core mechanism that drives the circadian rhythm is considered in this paper. Further, the delay model is phenomenological in nature rather than the precise description of all the underlying complex processes. In this way certain objectives are satisfied: (1) the complexity of the model is reduced due to delay, (2) the number of unknown parameters is reduced to a minimum, (3) the model has the ability to generate certain exotic dynamics such as quasiperiodicity and chaos that can be used to explain certain pathological features in terms of delay, and finally (4) the description of the phenomenological delay model in terms of Michaelis-Menten kinetics and Hill's equation for repression is close to a more realistic modelling of circadian rhythms than the electronic Van der Pol oscillators that are widely used for the description of the circadian rhythms. Also, in spite of abstracting and reducing the model to a smaller number of variables and parameters, most of the features are exhibited by the model. Thus the present model can be used as a more realistic phenomenological model for the description of mammalian circadian rhythms than models such as electronic Van der Pol oscillators that are widely used. So, the delay model studied here has three delayed positive and negative regulations, with the delayed positive feedback loops modelled by Michaelis-differential equations that include delays.

In this paper, following a recent trend toward the consideration of such delays, Sriram and colleagues introduce and explore a three-variable delay model for circadian rhythms in mammals. Rather than attempting a precise description of all the underlying processes, their model is phenomenological, and takes as dynamical variables the concentrations of three key proteins – BMAL1, PER-CRY complex and REV-ERBα. Their aim is to build a model with a small number of variables that is nevertheless capable of illustrating how delays in important feedbacks may underlie interesting features of real-world circadian rhythms. Among other things, their model exhibits 24-hour limit cycle oscillations, and natural entrainment to an alternative cycle of light and dark over the same period. Intriguingly, the model also shows quasiperiodic and chaotic oscillations under some conditions, in a way that is reminiscent of the breakdown of circadian regularity in certain health problems. This initial success, they suggest, demonstrates the promise of relatively simple models based around time delays.

The mammalian circadian circuit is so complex that researchers lack even a full list of its parts. Hence, there is as yet no way to build a complete model 'from the bottom up'. Work in this spirit has produced models with extensive sets of equations – 19 in one study, 73 in another – and will surely grow more complex with time. Detailed models of this kind have been used successfully to predict some features of the dynamics, such as the phase of entrainment to external stimuli and more detailed facts such as the time series of mRNA concentrations.

In contrast, other researchers have used 'coarse grained' variables to build simpler dynamical models that can still reproduce key qualitative aspects of the

Menten kinetics that describes saturation behavior, while delayed negative regulations are modelled with Hill's type of equation that describes a switch-like behavior. The form of interlocked positive and negative feedback loop is modelled along the same lines as that of Smolen et al. [8]. The role of delay in entrainment, in explaining mutant phenotypes, and in the occurrence of certain physiological disorders like sleep phase syndromes is addressed in this paper. Even though many of these aspects have been addressed earlier by other groups, the novelty in our model is exploring it in terms of delay.

The paper is organized in the following manner. In section 2, the recent work carried out on mammalian circadian rhythms and a short description of the biological circuit and mathematical model for the present work are given. Section 3 explains the choice of time lag used in the model equations. In section 4, the dynamics of free running oscillator, entrainment to LD cycles, phase response curves, and robustness to parameter variations are presented. In sections 5 and 6 the variation of delays and its implications in sleep syndromes are presented. The mutant phenotypes and the existence of multiple oscillatory mechanisms are presented in sections 7 and 8, respectively, followed by summary, conclusion and future direction of research.

Biological Circuit and Mathematical Model

Description of Earlier and Present Models for Mammalian Circadian Rhythms

In mammals, the transcription factors CLOCK and BMAL1 form dimers to activate the transcription of *Per* (period) and *Cry* (cryptochrome) genes. PER and CRY proteins, when they reach a critical concentration, attenuate the CLOCK/BMAL1-mediated activation of their own genes in a negative feedback loop. PER2 protein plays a positive role in *Bmal1* transcription thus establishing the positive feed-forward loop [11]. *Bmal1* expression is subjected to negative autoregulation through the product of the *Rev-Erbα* gene. *Rev-Erbα* itself is activated by BMAL1-CLOCK and negatively regulated by PER2 [12, 13].

The earlier ODE model by Leloup and Goldbeter [14, 15] for the circadian oscillations of mammals are based upon the interlocked positive and negative regulations exerted on *Per*, *Cry* and *Bmal1* genes by their protein products. Their model contains 19 coupled equations with 53 parameters and accounts for physiological disorders and the existence of multiple sources of oscillatory behavior. Recently, Forger and Peskin [16, 17] have developed a detailed, distinctly mammalian model by using the mass action kinetics with 73 differential equations. The parameters of their model, found by co-ordinate search method, accurately predicted the phase of the entrainment, amplitude of the oscillation and the time series of the mRNA and protein concentrations. Their model was also found to be robust to parameter variations and mutations. Becker-Weimann et al. [18, 19] have investigated the interdependency of the positive and negative feedback loop on the oscillation dynamics. They also proposed and predicted the occurrence of unexpected double mutant phenotypes.

In formulating the present model BMAL1 (B), PER-CRY (P) complex and REV-ERBα (R) protein concentrations are considered as the dynamical variables. The biological circuit is shown in figure 1. The transcriptional activators CLOCK and BMAL1 form a heterodimer which positively regulates *Per*, *Cry* and *Rev-Erbα* genes. In the model, only BMAL1 is considered as the dynamical variable because CLOCK protein does not oscillate and BMAL1 appears to be the limiting factor for the formation of dynamics. Here, Sriram et al. explore the importance of time delays, using a simple model that focuses on the concentrations of three proteins and a regulatory circuit shown in their figure 1. The authors have no way to 'derive' this circuit, but offer some motivating arguments for why these variables might offer a 'core' model of the process. For example, they argue that BMAL1 is the limiting factor in a crucial reaction that stimulates the production of both PER-CRY (a complex of two proteins) and REV-ERBα. The resulting equations for this circuit, their equations 1–3, reveal two positive and two negative feedback loops. These loops can be identified in the circuit by tracing the possible pathways on which one finds an even or odd number of '–' signs, as this indicates whether the overall loop tends to lead to increasing or decreasing levels of the proteins.

Most significantly, the model incorporates three time delays – δ_1, δ_2 and δ_3. The first implies, for example, that BMAL1 tends to lead to the production of PER-CRY, but only after a time delay of δ_1. As the authors readily admit, specific values for these delays cannot be measured directly in experiments. Rather, each is meant to reflect an effective delay brought about by the confluence of many processes, including 'translation, dimerization, phosphorylation, nuclear entry and posttranslational modification'. Based on extensive exploration of the model, seeking interesting dynamics similar to real circadian phenomena, they argue for plausible values of δ_1 = 13 h, δ_2= 6 h and δ_3 = 6 h.

The bulk of the paper examines the dynamics of the model under a number of important conditions. Using a standard set of parameters listed in their table 1, the authors first explored the model in the absence of any light stimulation, finding a 'baseline' oscillation with a period of 23.5 h (their fig. 2). In particular, figure 2b shows the limit cycle of the

BMAL1-CLOCK heterodimer [11]. PER-CRY complex is taken as another dynamical variable because PER and CRY expressions are positively coregulated by BMAL1-CLOCK [20]. Their phases are also similar. Furthermore, they both negatively regulate BMAL1 and CLOCK activity [21, 22], but the exact differential regulation of both *Cry* and *Per* genes in the core oscillator is not well known. In this model, the role of PER-CRY complex is 2-fold. First, PER-CRY complex binds BMAL1-CLOCK to indirectly repress its own production and that of *REV-ERBα*. Second, after complexation with BMAL1-CLOCK, it is assumed in our model that free PER-CRY complex activates both *Bmal1* transcription and also attenuates the production of REV-ERBα protein by regulating the *Rev-Erbα* gene. REV-ERBα, the negative regulator of BMAL1, is taken as the third dynamical variable. The repression of *REV-ERBα* is explicitly considered in the model, even though indirect repression is sufficient for modelling. Thus broadly, there are three negative and positive feedback loops, with BMAL1-CLOCK acting as positive limb and PER-CRY acting as negative limb [12]. The reduced model consists of three dynamical variables, namely BMAL1, PER-CRY complex and REV-ERBα proteins. The following are the corresponding delay differential equations:

$$\frac{dB}{dt} = \frac{v_s k_1^{n1}}{k_1^{n1} + R(t-\delta_3)^{n1}} + \frac{v_d P_f(t-\delta_2)}{k_2 + P_f(t-\delta_2)} - k_3 B$$

$$\frac{dP}{dt} = \frac{v_m k_5^{n2}}{k_5^{n2} + P_f(t-\delta_2)^{n2}} + \frac{v_p B(t-\delta_1)}{k_4 + B(t-\delta_1)} - k_6 P$$

$$\frac{dR}{dt} = \frac{v_r k_7^{n3}}{k_7^{n3} + P_f(t-\delta_2)^{n3}} + \frac{v_c B(t-\delta_1)}{k_8 + B(t-\delta_1)} - k_9 R$$

(1, 2, 3)

Here B and P are the protein concentrations of BMAL1 and CRY-PER complexes, while R is the concentration of REV-ERBα. P_f is the free PER-CRY complex ($P_f = P - B$) and $P_f = 0$ if $P < B$. The interlocked feedback loop of BMAL1 and PER-CRY complex is modelled in a similar way to that of Smolen et al. [8] to achieve three variables B, P and R, that is, the trajectory of a point with coordinates $B(t)$, $P(t)$ and $R(t)$. Importantly, this behavior is in most cases qualitatively robust to changes in the parameter values of about 10%, as shown in figure 3. Of particular note, variations in the delays δ_1, δ_2 and δ_3 alter the amplitude and period of the circadian rhythm, as displayed in more detail in the authors' later figure 5. One qualitative conclusion that emerges from these studies is that changes in these delays generally have more influence than do changes to other parameters in the model.

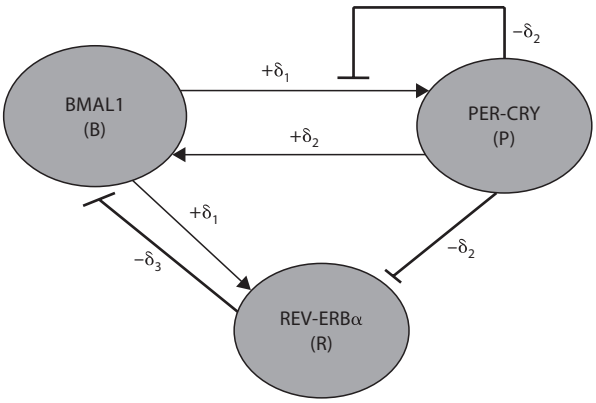

Fig. 1. Schematic representation of the present model for the mammalian rhythm. B, P and R are the BMAL1 protein, PER-CRY complex, and REV-ERBα proteins, respectively. δ_1 is the delay in the positive feedback from B to initiate the synthesis of PER-CRY protein. The delay δ_1 is also the delayed positive feedback from B to initiate the synthesis of REV-ERBα protein. δ_2 is the time delay for the PER-CRY protein to activate and suppress BMAL1 and REV-ERBα protein, respectively, and to suppress its own production. δ_3 is the time delay for REV-ERBα protein to suppress the production of BMAL1. See text for further details.

Baseline oscillation, of course, is only one aspect of real-world circadian circuits. To be useful, the model should do much more, and the authors go on to show that it can. Biologists know that the phase of the mammalian circadian cycle can be altered by short intervals of illumination, especially at nighttime. Various mechanisms make the circadian chemistry sensitive to light, which can influence the concentrations of particular proteins, pushing the biochemical cycle to a different phase. To explain this effect, Sriram and colleagues note that the parameter v_m sets the rate of production of the PER-CRY complex. In mammals, light should stimulate such production because it increases the rate of transcription of the *Per* gene. So v_m is the proper 'handle' to model light stimulation in the circuit. Hence, they ran a series of simulations in which v_m remained at a base value of 0.7 always, except for an interval of 1 h when it jumped to 1.0, reflecting a virtual 1-hour burst of light applied somewhere in the cycle. The results (fig. 4a) show biologically realistic behavior, with the phase of the cycle changing by up to 4 h.

As the authors admit, however, the model is not perfect. As it turns out, mammals do not show any phase sensitivity if light is applied during certain 'dead zones' of the cycle; an hour of extra

count for the interdependency of BMAL1 and PER-CRY complex proteins. $v_{s,p,r,c,d,m}$ are the rates at which the proteins are synthesized and the production rate v_m of PER-CRY complex increases in the light phase. The model has two positive and negative feedback loops. The delayed negative feedback loop of PER-CRY complex is interlocked with a delayed positive feedback loop of BMAL1. The other parameters are the Michaelis constants $k_{1,2,4,5,7,8}$, the Hill's coefficients, $n_{1,2,3}$ characterizing the degree of co-operativity of the repression processes; $k_{3,6,9}$ are the first-order degradation constants of *B*, *P* and *R*, respectively.

The present model differs from other models described earlier as regards the following aspects: (1) Proteins are only considered as dynamical variables with delay incorporated in them. (2) The delay in the model is extracted from the experiments and the phosphorylation and dimerization reactions are not explicitly considered. The delays $\delta_{1,2,3}$ are introduced in the model to signify the time taken for translation, dimerization, phosphorylation, nuclear entry and post-translational modification, as the number of phosphorylation reactions in the dynamical variables is not exactly known, but appears to be high. Therefore the number of variables and parameters chosen are considerably fewer than in the rest of the models. (3) Many pathological features, like sleep phase syndromes and cancer incidence are explained in terms of delays. (4) Finally, the model has an equal number of positive and negative regulations, with positive regulation modelled by Michaelis-Menten kinetics, whereas the negative regulations are modelled by Hill's type of equation. Each equation thus has two terms: a delayed positive reulation and a delayed negative regulation.

Choice of Delays and General Remarks

The choice of delays in the model is based on the following reasoning. It has recently been reported by Tamura et al. [23] that the nucleocytoplasmic shuttling and phosphorylation states of BMAL1 are regulated by the circadian clock, and this temporally regulated and time delayed nuclear entry of BMAL1 is important for the maintenance of a stable oscillating clock. The time delay is in the order of 12–13 h, which is much more than the time delay between *Per1*/*Per2* transcription and PER1/PER2 protein accumulation in the nucleus, which is approximately 6–9 h [24–27]. For the present model the time delays are taken to be 13 h for BMAL1 activation of *Per*, *Cry* and *Rev-Erbα* genes, 6 h for *Per* activation of *Bmal1*, suppression of *Rev-Erbα* and its own product, and finally 6 h for REV-ERBα to suppress *Bmal1* transcript, even though its exact time delay is not known. All the cellular processes occur at very short time scales while circadian clock processes occur at long time scales. An intuitive explanation can be given for the occurrence of 24 h circadian oscillations in terms of delay. In our model, the overall time delay for the positive and negative regulation is approximately one circadian cycle. Delayed BMAL1 activation (13 h) of PER-CRY complex and REV-ERBα constitutes half of the circadian cycle. Delayed activation and suppression of BMAL1 and REV-ERBα, respectively, by PER-CRY complex and its own suppression (6 h) constitutes quarter of the cycle. Since the time delay for REV-ERBα repressing the BMAL1 is not known, it is assumed to be 6 h, which is another quarter of the circadian cycle. In totality, half of the circadian cycle amounts to a delay in positive regulation (BMAL1 activation) and another half is the negative regulation (PER-CRY and REV-ERBα put together). The interplay light in mid afternoon typically has no effect, for example. In the model considered here, the phase shift observed during the day is small, yet the dynamics do not show any dead zones.

Another central feature of the mammalian circadian clock is its sensitivity to entrainment by the periodic light-dark cycle. The model also shows this behavior, as demonstrated in figure 4b. Without any light, the cycle runs with a period of 23.5 h, which then changes to 24 h to match the cycle of the external light. Interestingly, the authors also point out that small changes in the delay δ_2 induce a shift in the phase of the circadian clock, and in a way that suggests the symptoms of common sleep disorders. Individuals with 'advanced' or 'delayed' sleep phase syndromes find their sleep-wake cycles slightly off from the natural light cycle, making it difficult to sleep at night or to wake in the morning, for example.

A third interesting feature of the model is its ability to show irregular dynamics for other values of the delay δ_2, again in the presence of a periodic light-dark cycle. In some cases, despite the regularity of the light cycle, the circadian clock never falls into a steady rhythm, but behaves erratically instead. In particular, chaotic dynamics is shown in the authors' figure 6. Figure 6a is a bifurcation diagram showing the maximum value of the variable *P* for different values of the delay δ_2. Clearly, the nature of the dynamics is in many cases extremely sensitive to small changes in δ_2. More importantly, the protein concentrations for some values of δ_2 vary with a broad range of component frequencies (as shown in the power spectrum in fig. 6d), a classic sign of chaos and highly irregular behavior. Nonchaotic but quasiperiodic behavior is revealed in figure 7. As the authors point out, such irregular dynamics and lack of entrainment to the light-dark cycle have been observed in some blind people, for whom one might naturally expect the en-

of delayed positive and negative feedback loops contributes to one circadian cycle.

Dynamics of Free Running System at Constant Darkness

In our model there are overall 21 parameters, including the three delays. Among the three delays, $\delta_{1,2}$ are essential for circadian oscillations (see sections 7 and 8 for variations in delays). A standard parameter set (table 1), chosen by trial and error variations, is used in simulation under constant darkness (DD). The parameter values are also chosen based on other constraints set by experimental observations such as the ability of the model to explain entrainment curves, mutant phenotypes and certain physiological disorders. In our model, the oscillations of BMAL1 and PER-CRY complex are approximately antiphase to each other as observed in the experiments [13]. The period of the oscillation is 23.5 h (fig. 2a, b). The model exhibits oscillations when stochastic simulations are performed by Gillespie's algorithm [28] (fig. 2c) and appears to be robust to internal noise (see section 8 for discussion). The methodology followed for internal noise simulation using Gillespie's algorithm is the same as that of Smolen et al. [8].

The period and amplitude of circadian rhythms should be robust to small variations in the parameters. In this study, the standard set of values (table 1) was chosen initially and each individual parameter is then changed by $\pm$10% of the standard parameter set. The amplitude was measured as the peak to trough concentration difference of PER-CRY protein. The corresponding scatter plot is shown in figure 3. The period and amplitude of the free running rhythm of PER-CRY protein are 23.5 h and 1.132 nM respectively, under DD. To identify the sensitivity of the parameters in each equation, the parameters are divided into four groups, namely parameters in the positive feed back loops, negative feedback loops, degradation rates and finally the three delays. Oscillations are not preserved in all the simulations and the appearance of the oscillations did change from the controlled oscillations for some of the parameters. Dampened oscillations are obtained for parameters v_d and k_2 that are present in the interlocked feedback loops. Dampened oscillations are also obtained when δ_2 lies between 5.4 and 5.9 h, that falls in the –10% range, but between 3–5 and 6–16 h robust oscillations are obtained (see further details of delay variations in section 5). Delays $\delta_{+1,+2}$ (here, +1 and +2 are +10% changes in the parameter in delay δ_1 and δ_2), are most sensitive to small changes and the period and amplitude varied by more than 45%. The rest of the parameters varied by only 30% in response to the 10% training link to the light-dark cycle to be weak. They also suggest that such 'wandering' rhythms may be linked also to the incidence of some cancers.

The authors finally show also how the model can account, in qualitative terms, for variant phenotypes seen in several mammalian mutants. Mutations affect key proteins in the biochemistry of circadian rhythms, and can easily destroy such oscillations altogether. But it does not require detailed molecular models to explore such effects, which may arise from secondary changes in the delay parameters. The authors show in several cases how these parameters seem to offer a 'natural' level for explanation, as parameter changes lead to significantly altered phenotypes of the kind seen in common mutations.

Again, this illustrates that the delays δ_1, δ_2 and δ_3 seem to be highly relevant variables. Establishing this basic point, in fact, may be the most significant achievement of this paper, and suggests that further focus on the role of delays may lead to rapid advance in our understanding of circadian rhythms in many organisms.

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Table 1. Parameter values for the free running rhythms for three-variable delay model of mammalian circadian rhythm

Parameter	Value used in simulation
v_s	4 nM h^{-1}
v_d	0.97 nM h^{-1}
v_p	1.0 nM h^{-1}
v_m	0.7 nM h^{-1}
v_r	0.1 nM h^{-1}
v_c	1.0 nM h^{-1}
k_1	0.5 nM
k_2	2.0 nM
k_3	0.21 h^{-1}
k_4	0.9 nM
k_5	0.6 nM
k_6	0.45 h^{-1}
k_7	0.1 nM
k_8	0.1 nM
k_9	0.45 h^{-1}
n_1	2.0
n_2	2.0
n_3	2.0
d_1	13 h
d_2	6 h
d_3	6 h

variation (fig. 3). Among the parameters in the negative and positive feedback loop, the parameters in the positive feedback loops are highly sensitive. The degradation rates are also sensitive to changes, but not as sensitive as delay or other parameters in positive feedback loops. To summarize this section, the sensitivity of parameters that are divided into four groups can be ordered as follows: delay > positive feedback loops > degradation rates > negative feedback loops.

Phase Response Curves

Phase response curves are obtained by perturbing for 1 h the PER-CRY complex production rate v_m as described below. In mammals, light induces *Per* transcription more strongly than it affects PER protein degradation. As we have considered only proteins as dynamical variable, it is assumed here that the PER protein concentration increases proportionately with the increase of *Per* mRNA transcript in the presence of light. In simulation, the initial phase of 0 h of the unperturbed rhythm corresponds to the minimum of PER-CRY protein concentration. This is taken as the beginning of the subjective day.

In mammals, light pulses cause phase delays in the early subjective night, phase advances in the late subjective night, and no phase shifts during the subjective day [29]. In simulation, the light-sensitive parameter v_m is applied as a square wave pulse of basal value 0.7 and maximum of 1.0 for 2 h, and the perturbation is started at the minimum of PER-CRY protein concentration. When the light pulse is applied at and near the minimum, the phase of PER-CRY oscillation is advanced. A phase advance of up to 4 h is obtained between the phases 0 and 7 h. Phase advance is also seen in the late subjective night between the phases 22–23.5 h (fig. 4a). No explicit dead zone is obtained in our delay model, but the phase shifts during subjective day are smaller. Phase response curves have also been obtained for different durations and intensities (not shown), but the shape of phase response curves remained the same. At present it is not known why dead zones are not observed in our model. Recently, Geier et al. [30], in their model for mammalian circadian rhythm, have found no dead zones during subjective day, but when phase-dependent gating terms were included in their model, they observed dead zones. It may thus be interesting to suitably incorporate gating terms in our models as delayed variables to study the nature of phase response curves. To conclude this section, no dead zones are obtained during the subjective day, but phase delays are observed during

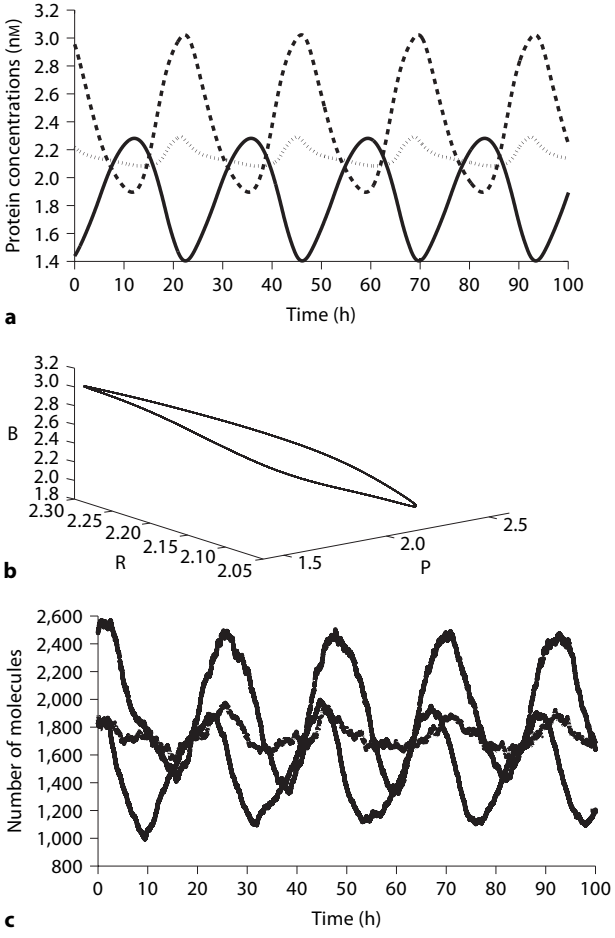

Fig. 2. a Sustained oscillations generated by the model. The BMAL1 protein (black continuous line) is approximately in antiphase to proteins PER-CRY protein (black dotted lines) and REV-ERBα (black dashed-dotted lines). The time series were obtained by numerical integration of delay equations 1, 2 and 3 under DD for the standard parameter set (table 1). **b** The limit cycle in the model of circadian oscillations corresponding to **a**. All the simulations were performed using a fourth-order Runge-Kutta algorithm, with a step size of 0.01 h. **c** Robustness of mammalian circadian rhythms to internal noise and time courses of different concentrations.

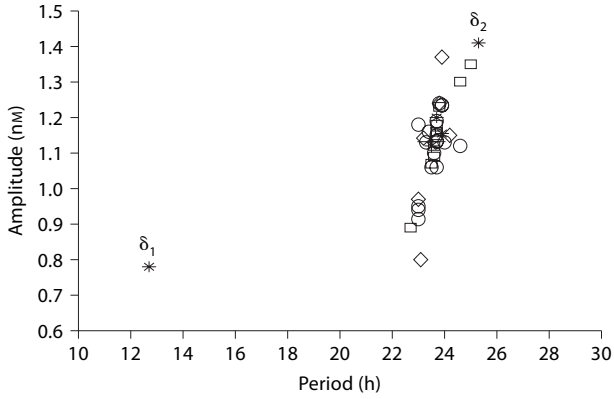

Fig. 3. Robustness of mammalian model to parameter variation. The scatter plot displays the period and amplitudes of circadian oscillations for ±10% variation in parameter. The symbol + denotes the amplitude and free running period in DD. Different symbols are used to denote the parameters that are involved in feedback loops; delays and degradation constants are positive feedback loops (□), negative feedback loops (○), delays (*), and degradation constants (◇).

the early subjective night and phase advances during the late subjective night.

Entrainment to Periodic Variation: Explanation for Sleep Disorders in Terms of Delay δ_2

Periodic external stimulations by light are studied by periodically switching on and off the light sensitive parameter v_m. The entrainment of the oscillator is found to occur to external periodic stimulation of 12:12 LD cycles (fig. 4b). The parameter is set to a low value ($v_m = 0.7$) during the dark phase and is increased up to a higher value ($v_m = 3$) during the light phase. After all the transients have died down, the system is found to be phase locked to the external stimulus, which is 24 h, whereas the autonomous oscillations in DD are 23.5 h. In our model the rising and falling phase of PER-CRY complex partly coincides with the LD cycles. The entrainment of the oscillator is found to be sensitive to delay. The delay δ_2 is chosen as the variant because it is presumed that the changes in the concentration of PER-CRY complex caused by light will correspondingly affect the changes in the delay δ_2 in the feedback. When the delay δ_2 is changed from 6 to 8 h for the same LD values, the phase of the oscillator is delayed with the maxima shifting by around 2.6 h with respect to the entrained curves for the normal set of parameter values. The rising and falling phase of the oscillators coincides well with the LD cycle. This may be due to the interplay of production rate v_m and the delay δ_2. Light enhances the production of *Per* transcript and thereby increases PER concentration. However, the transcription is attenuated by the autoregulatory feedback loop of PER-CRY complex. For large delays (8 h) the transport of PER-CRY complex to the nucleus is slower and correspondingly the attenuation too is slower. This causes the PER-CRY complex to fall slowly. The reverse is the case when the delay takes on a smaller value.

The variation of phases with the changes in delay δ_2 can be related to sleep syndromes. The advanced sleep phase syndrome (ASPS) is characterized by evening sleepiness with the onset of sleep much earlier than desired. In humans, this disorder is associated with the mutation in PER2 phosphorylation sites that induces a faster accumulation of PER2, an acceleration of clock feedback loops, shortened circadian period and advanced phase shifting [31]. The delayed sleep phase syndrome (DSPS) is characterized by an inability to fall asleep or to awake spontaneously at the desired times. It is associated with sleep onset and wake times that are intractably later than desired. It has been proposed [32] that alteration in CKIε phosphorylation of *hPer3* gene may be responsible for the DSPS syndrome that results in phase delay. In our model, when the delay δ_2 is increased or decreased under 12:12 LD cycles the phase is advanced or delayed, respectively, which accounts for ASPS and DSPS. Further implications of delays in both DD and LD are discussed in the next two sections.

Effect of Variation in the Delays δ_1, δ_2 and δ_3 under DD

As explained in the earlier sections, one of the processes that is lumped in the form of delay in our model is that of phosphorylation reactions. Phosphorylation is one of the most common types of protein modifications that has been implicated not only in determining protein stability, but has also been shown to regulate different cellular activities [33–35]. It is found that the variation of all the three delays produced variations in the period (fig. 5). Oscillations are not obtained in the absence of delay δ_1 or δ_2 and in its absence the variation in parameters did not bring about any oscillations. Oscillations are obtained in the absence of delay δ_3, but the period and amplitude varied. As the delay δ_1 or δ_2 is varied, keeping all the other parameters constant, the period also varied accordingly, but once the delay δ_2 is decreased to a certain value (5 h), the period of the oscil-

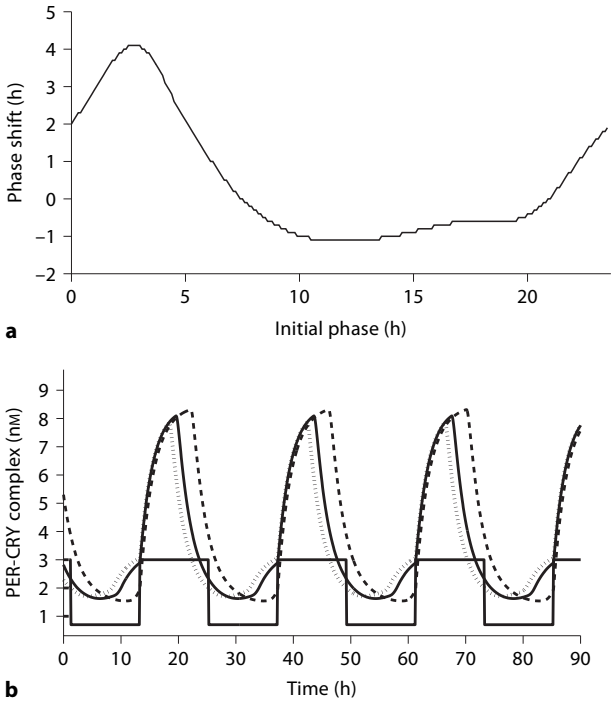

Fig. 4. Phase response curves and entrainment curves. **a** Light-induced phase shift obtained theoretically for perturbation applied to the oscillator. The perturbation takes the form of a 1-hour-long increase in the production rate of PER-CRY protein complex concentration, ν_m from 0.7 to 1, triggered by light. Circadian time 0 h is taken as the minimum of PER-CRY protein complex. **b** Entrainment by LD cycles: the oscillations show the influence of the magnitude of change of the light-dependent parameter ν_m for 12:12 LD cycle. LD cycle is a square wave, in which ν_m steps up from 0.7 to 3. The continuous line is the entrained PER-CRY protein complex, where the curve rises in the light phase and falls in the dark phase and the period is 24 h. The dashed-dotted line is the phase advanced curve, when delay δ_2 is decreased to 4 h. The dashed line is the advanced curve when delay δ_2 is increased to 8 h.

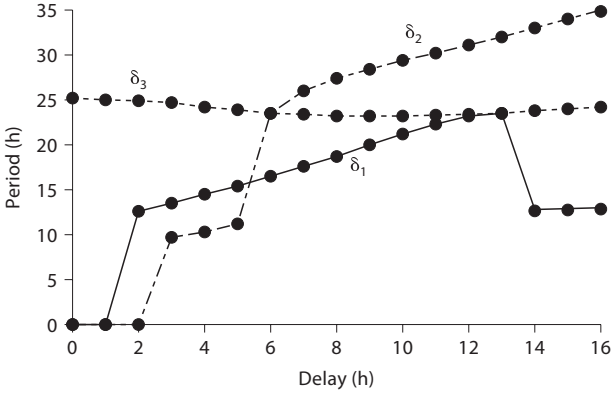

Fig. 5. Variations of the period in the mammalian model to delay variation. The plot displays the effect of each delay on the period of the oscillator under DD, keeping all the parameters constant. The lines are drawn only for visualization. See text for further explanations.

lator abruptly decreases. This may be because PER-CRY complex proteins enter the site of action very rapidly and consequently its own repression as well as that of REV-ERBα takes place rapidly. This causes a considerable decrease in the concentration of all the proteins, resulting in a much shorter period. Also the complexation of the PER-CRY complex with BMAL1 takes place rapidly thus not allowing it to efficiently activate *Per*/*Cry* and *Rev-Erb*α genes. The amplitude of the PER-CRY complex decreases by 75% in comparison to normal free running rhythm. When delay δ_2 takes on values between 5.4 and 5.9 h, dampened oscillations are obtained whereas between 3–5 and 6–16 h robust oscillations are obtained. In the delay range between 5.4 and 5.9 h, the oscillations are retained when delay time is compensated by the delay δ_1 of the positive feedback loop. For example, when the delay δ_2 is changed from 6 to 5.4 h (difference of 0.6 h with respect to standard type), dampened oscillations are obtained, whereas when the time lag δ_1 in positive feedback loop is increased from 13 to 13.6 h, oscillations are retained, but the period and amplitude deviate largely from the circadian period. In other words, if the negative feedback is compensated by the positive feedback delay, oscillations are retained. However, other parameters may also bring about oscillations, but the compensation by delay appears to be most favorable. This suggests that there is some compensation by other feedback loops that keeps the oscillations in the system intact. The effect of changing δ_1 also brings about large changes in the period when the delay is lowered to 2 h or increased to 14 h. The delay also brings about various dynamical changes and in the next section the effect of varying delay δ_2 under periodic LD cycles and its implications in physiological disorders are discussed.

Effect of Delay δ_2 and Periodic Forcing by LD Cycles: Explanation for Non-24-Hour Sleep Syndrome and Cancer Incidence

Most of the delay models that have been proposed so far for the circadian rhythms are found to exhibit only periodic oscillations, which are limit cycles in nature. However, for *Drosophila*, the ODE model proposed by Leloup et al. [2, 3] was found to exhibit autonomous chaotic oscillations for a narrow parameter regime under constant DD conditions. Nonautonomous chaos has also been obtained in conditions of periodic forcing by LD cycles in the case of *Neurospora* [36]. Nonautonomous chaos is observed in our three-variable delay model in the conditions of constant periodic forcing by LD (12:12) cycles and by varying the delay δ_2, keeping other parameters constant with δ_1 taken to be 12 h. The light-controlled parameter v_m takes a square wave function from 0.7 to 1.0. For this intensity, the delay δ_2 is varied over a wide range of values. Apart from limit cycle oscillations, quasiperiodic and chaotic oscillations have also been observed in the system (fig. 6). It is well known that the lack of entrainment in the 24-hour sleep-wake cycle is linked to a physiological disorder [37]. The non-24-hour sleep-wake syndrome and lack of entrainment are more frequently observed in blind patients. A period of long days with 24–40 h without sleep followed by 14–24 h of uninterrupted sleep occurs in blind patients. This amounts to a lack of entrainment of the circadian cycle to the LD cycles. The lack of entrainment to the LD cycle is simulated in our model by varying the delay δ_2 of PER-CRY complex under the influence of the 12:12 LD cycle. Even though mutation screening analysis of human *Per3* and *Clock* genes have not yet revealed an association with the non-24-hour sleep-wake syndrome, there is a possibility that this may be the cause of the syndrome [32]. It is shown here that the occurrence of the disorder in a constant 12:12 LD cycle by varying the delay δ_2 in the feedback loop of PER-CRY complex gives rise to quasiperiodic oscillations. The quasiperiodic oscillations are obtained when delay δ_2 is increased to 8 h (fig. 7). The lack of entrainment to LD cycles points to the fact that the delay in the PER-CRY complex to reach the site of action under the LD cycle leads to the loss of entrainment. The disorder may be due to the lack of an improperly delayed negative feedback from the PER-CRY complex, even though it is not established experimentally.

Chaos is also obtained for certain delay values and one such case is shown in figure 6, when the delay is reduced to 3 h. The power spectrum has a broad range of frequencies, which supports the occurrence of chaotic phenomena. The chaos in circadian alterations can be linked to cancer incidence, as recent animal studies have shed light on the links between circadian rhythm and cancer incidence. Mice with mutations in the *mPer2* gene are prone to tumor development and early death [38]. It has been found that the circadian rhythms of patients afflicted with cancer exhibited diminished amplitude, phase shifts, abrupt period changes and erratic peaks and troughs [39]. These data suggest that the chaos in circadian rhythms may serve as a marker for tumor status.

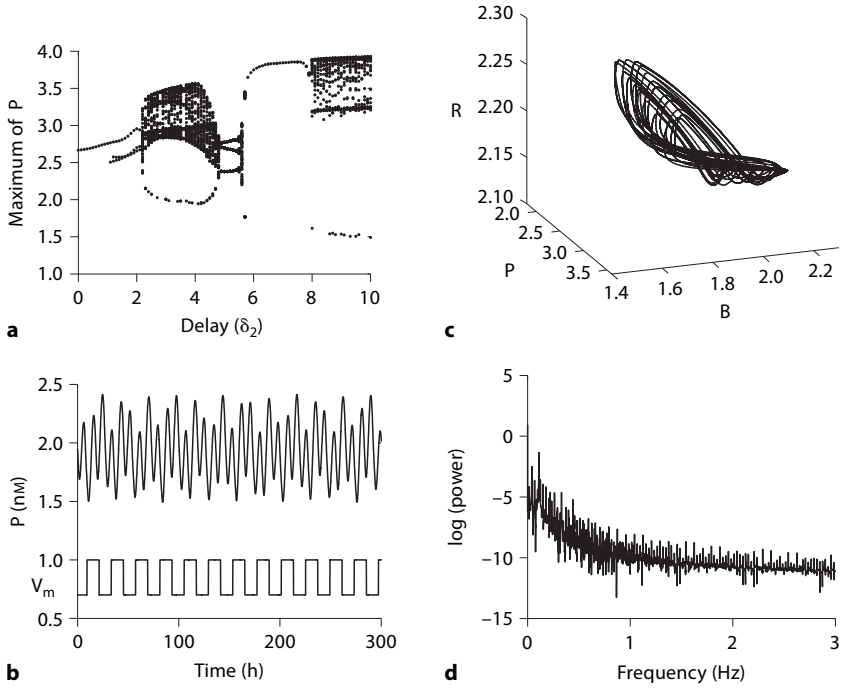

Fig. 6. a Bifurcation diagram obtained for the constant LD cycle with delay δ_2 as the parameter. The 12:12 LD cycle simulated with v_m taken as square wave function that is changed from the basal value of 0.7 to 1. **b** Chaotic time series of dynamical variable P, with 12:12 LD cycle for delay δ_2 = 3 h. **c** Chaotic attractor. **d** Power spectrum. P is the dynamical variable, namely PER-CRY complex. All the other parameters are kept constant with delay δ_1 taken as 12 h.

Simulation of Mutant Phenotypes

Simulation of Mutant Phenotypes by Variation in Delay

Variations in delays δ_1 and δ_2 of positive and negative feedback loops are used to simulate the gene mutant phenotypes. In experiments, mutation in *Bmal1*$^{-/-}$ results in complete loss of oscillations and the phenotype is arrhythmic. This is simulated by setting δ_1 to 0 and therefore BMAL1, PER-CRY complex and REV-ERBα reach a very low concentration. The mutation in *Per2* gene is simulated based on the following reasoning. In our model, we have not separately considered *Per* and *Cry* genes and have taken the PER-CRY complex as one dynamical variable. The role of PER2 is 2-fold: to activate *Bmal1* and repress *Rev-Erb*α. PER2 being a weak inhibitor, it is assumed here that its main role is to activate *Bmal1* transcription. Mutation in *Per2*, namely *Per2*^{Brdm1} results in low *Bmal1* transcription and protein concentration. This can be simulated in our model by either one of the following ways: (1) by setting δ_2 to 0 by assuming that the mutation affects one of the delayed feedback processes, (2) by decreasing the inhibitory constant k_5 of PER-CRY complex onto itself and finally (3) by decreasing the inhibitory strength k_7 of PER-CRY complex on REV-ERBα. Since PER2 is the weak inhibitor, the inhibitory strength of PER-CRY complex on REV-ERBα is weak and should not have much effect when mutated. It turned out to be so in our model as varying the inhibitory strength k_7 of PER-CRY complex on REV-ERBα did not quench the oscillations. On the other hand, when delay δ_2 is set to 0, oscillations are quenched, with BMAL1 reaching very low concentrations. This is also true when k_5 is varied, which suggests that PER2 plays a more important role in *Bmal1* transcription than in repressing Rev-Erbα.

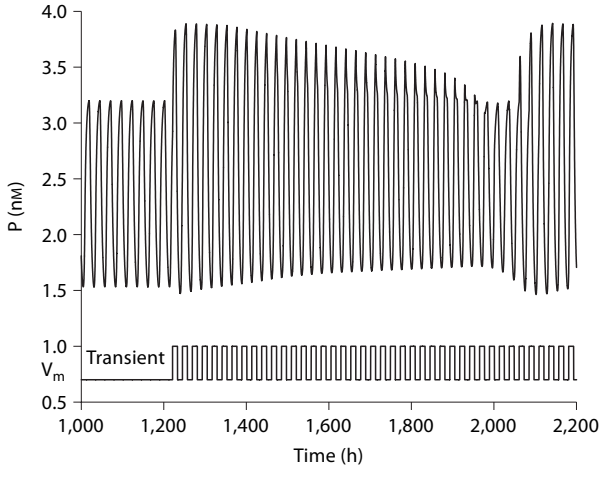

Fig. 7. Time series of quasiperiodic oscillations obtained as the effect of LD cycles for the delay δ_2 = 8 h. The delay δ_1 is taken as 12 h. v_m is a square wave, changing from the basal value of 0.7 to 1.

Interplay of Positive and Negative Feedbacks: Plausible Explanation for the Observation of Per2^{Brdm1}*/Cry2*$^{-/-}$ *Double Mutants*

Reppert and Weaver [13] proposed that PER2 positively regulates *Bmal1* transcription and CRY inhibits PER2/CRY transcription. *Per2*^{Brdm1} mutation results in low *Bmal1* transcription and results in complete loss of oscillations. The second mutation in *Cry2*$^{-/-}$ leads to the rescue of oscillation. The occurrence of the double mutation that results in the rescue of oscillations is due to the interplay of positive and negative feedback loops in the system. In our model we did not explicitly consider PER and CRY as separate dynamical variables, but as a complex and this cannot explain the occurrence of double mutation directly. Thus to explain this aspect, we considered the combined effect of interlocked positive and negative feedback that occurs in the model. Also, we utilized the sensitivity of the term v_d that occurs in the PER-CRY complex positive feedback on BMAL1 and k_5, the inhibitory strength of negative feedback on itself. Decreasing the strength of positive feedback quenches the oscillations, whereas the oscillation is rescued by decreasing the inhibitory strength in the negative feedback (fig. 8). There is no variation in the amplitude and the period of the oscillations is 22.5 h, which is close to the period of wild type.

We also simulated the *Rev-Erb*$\alpha^{-/-}$ mutant phenotypes, where in the experiments, *Bmal1* mRNA and protein do not oscillate but are expressed at constant high levels and oscillations of *Per* mRNA and protein remain stable. To silence REV-ERBα protein the terms v_c, v_r and delay δ_3 are set to 0.1, 0 and 0, respectively. For high BMAL1 concentrations, the threshold term k_2 in the positive feedback of equation 1 is set to 100. For PER-CRY complex to oscillate, the term v_m in equation 2 is set to 7. From these param-

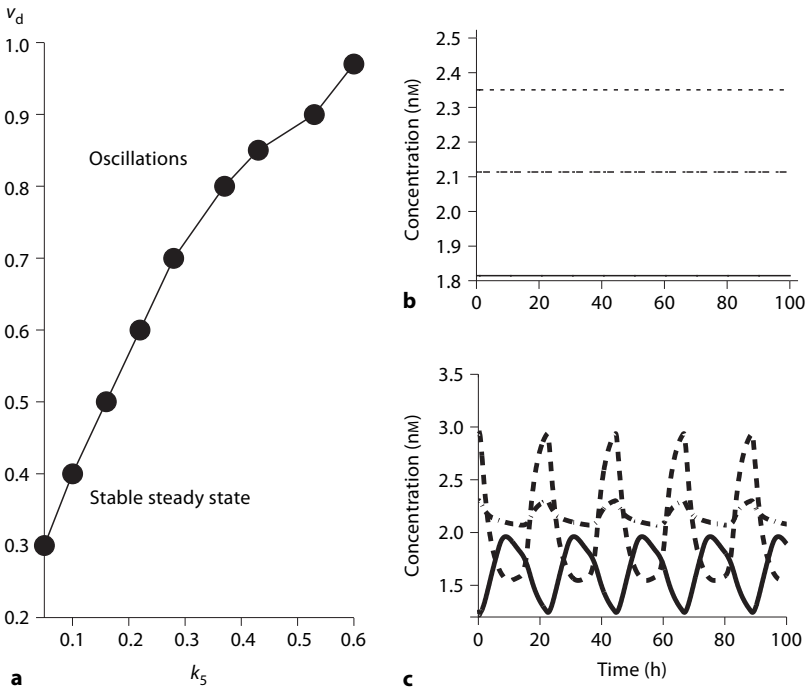

Fig. 8. a Variation of positive feedback ν_d and the inhibitory strength k_5 of PER-CRY complex. The inhibitory strength k_5 is varied to simulate $Per2^{Brdm1}/Cry2^{-/-}$ mutation and the demarcation between oscillatory and steady state are plotted. **b** One particular case is chosen to show the loss of oscillation due to $Per2^{Brdm1}$ mutation, when ν_d is reduced from 0.97 to 0.6. **c** Rescue of oscillations due to $Cry2^{-/-}$ mutation, when k_5 is reduced from 0.6 to 0.2.

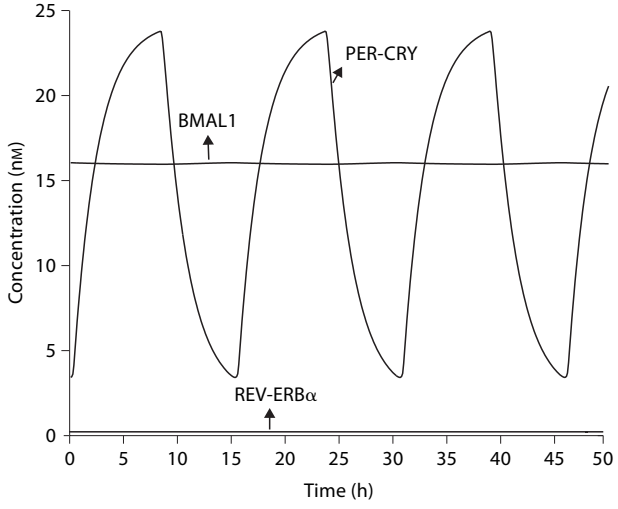

Fig. 9. Simulation of $Rev\text{-}Erb\alpha^{-/-}$ mutation. Stable oscillations in PER-CRY complex protein concentration, high level of BMAL1 and low level of REV-ERBα concentration when ν_c, ν_r and delay δ_3 are set to 0.1, 0 and 0, respectively, k_2 in the positive feedback of equation 1 is set to 100 and finally ν_m in equation 2 is set to 7. See text for explanations.

eter values it can be seen that feedback loops operating on REV-ERBα are silenced by decreasing the effects of positive and negative feedback loops from BMAL1 and PER-CRY complex, respectively. In our model, this leads to a nearly constant expression of BMAL1, a low expression of REV-ERBα and stable oscillations in the PER-CRY complex (fig. 9). The period of the oscillation is found to be 15.1 h, which deviates (64%) from the circadian oscillations.

Simulation of $Cry1^{-/-}$ and $Cry2^{-/-}$ Mutants

It is well documented in the literature that the null mutations of *Cry1* and *Cry2* have two different effects on the period of the oscillation. The period of $Cry1^{-/-}$ is shorter, while that of $Cry2^{-/-}$ is longer [40]. This is due to the different inhibitory strengths of *Cry1* and *Cry2* mutants. Also, the double mutation $Cry1^{-/-}/Cry2^{-/-}$ results in complete loss of oscillations. In our model, the variation in inhibitory strength k_5 of PER-CRY complex results in an increase or decrease in the period of the oscillations (fig. 10), even though the choice of this parameter is arbitrary. From figure 10 it can be seen that when k_5 is decreased with respect to the standard value (0.6), the period decreases initially, followed by an increase, which corresponds to the behavior in $Cry1^{-/-}$ mutants. When k_5 is increased with respect to the standard value, the period also increases and reaches a saturation. This corresponds to a $Cry2^{-/-}$ mutation. When k_5 is greater than 0.8 the system reaches a stable steady state. This corresponds to double $Cry1^{-/-}$ and $Cry2^{-/-}$ mutant phenotypes. The variation in inhibitory strength accounts for both phenotypes. It is of interest to see the effect of delay δ_2 in explaining *Cry* single and double mutant phenotypes, which will be done in a future study.

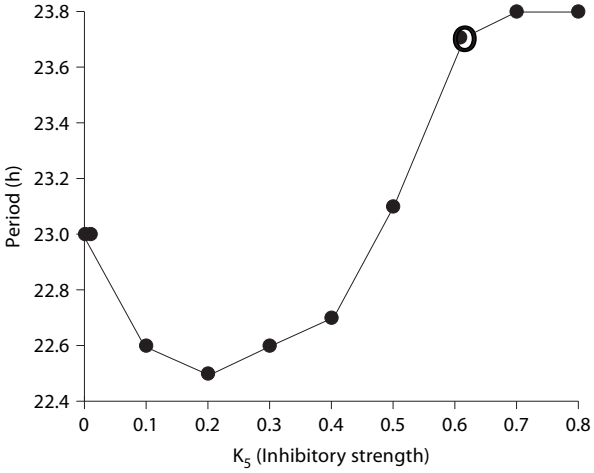

Fig. 10. Variation of period in the inhibitory strength of PER-CRY complex feedback. The inhibitory strength k_5 is varied to simulate $Cry1^{-/-}/Cry2^{-/-}$ mutation and the corresponding changes in the period are plotted. The circle denotes the period for the standard parameter set.

Summary, Conclusion and Future Direction of Work

In this paper, we have proposed a three-variable delay model with effectively three time delays to describe the circadian oscillations of mammals. The present model is based on a representation of a delayed positive feedback loop interlocked with a delayed negative feedback loop of transcription regulation. The dependent variables are taken to be the concentrations of BMAL1, PER-CRY complex and REV-ERBα proteins. The interlocked positive and negative feedback loops are modelled according to Smolen et al. [8].

The numerical simulations fulfill most of the characteristic features of the circadian oscillations. The oscillations have a circadian period and show stable limit cycle behavior. BMAL1 oscillations are approximately antiphase with PER-CRY and REV-ERBα protein oscillations as observed in the experiments. Most of the parameters are fairly robust to small changes, but some parameters are extremely sensitive to small changes. To see the effect of changes the parameters are grouped into four types. Delays are the most sensitive parameter, as both amplitude and period varied drastically for the 10% change in the values. This is followed by the parameters in the positive feedback. Instability and loss of robustness in the model are caused mostly by the parameters in the positive feedback loops.

The novelty of the model is that an attempt has been made to explain many of the characteristic features of circadian rhythms in terms of delays that are known from the experiments. Also, to keep the model simple, only protein interactions are taken into consideration and this reduces the number of parameters and variables to a large extent in comparison to previous models proposed by others. The model shows entrainment to both shorter and longer LD cycles. In all the LD cycles, the oscillator is entrained to a 24-hour rhythm for the standard parameter set. When delay δ_2 is varied under LD cycles, the model exhibits phase advance, phase delay and lack of entrainment, which are linked to physiological disorders. Apart from limit cycle, quasiperiodic and chaotic oscillations are also observed when the delay δ_2 is varied under the influence of constant periodic 12:12 LD cycles. Periodic forcing is known to bring about rich dynamical phenomena [41] and in our model constant periodic forcing with delay brings about a rich bifurcation diagram. The observed complex phenomena such as quasiperiodic and chaotic oscillations are linked to non-24-hour sleep-wake syndrome and occurrence of cancer incidence, which may be a direct consequence of improper delayed circadian regulation due to *Per* gene mutation. The effects of mutant phenotype on the circadian period are well simulated by changing the parameters and time delay. The model also uncovers the possible existence of multiple oscillatory network.

Recently, we have been concentrating on various other aspects in the delay model of the mammalian circadian rhythms. One of the most important aspect is that of simulating the effect of internal or molecular noise in the system. Many molecules that control the regulation of transcription act at an extremely low concentration and this causes large variations in the oscillations [42]. In order to simulate the effect of fluctuations, the Gillespie algorithm for Monte Carlo simulation is employed [28]. The model exhibits oscillations for high molecule numbers (fig. 2c), but the effect of noise at very low molecule numbers has not been fully explored. It is pertinent to point out here that Gillespie's algorithm is not technically correct to apply for delay differential equations. Gillespie's algorithm assumes that the events in the reactions are Markovian in nature. In the presence of delay, this is no longer valid as the reaction events in the delay equa-

tion assume a non-Markovian character. As there is no equivalent for Gillespie's algorithm developed for the system of delay equations at present, we have proceeded with Gillespie's algorithm. We are now exploring for alternative algorithms to simulate the internal noise for the system of delay equations.

There are also certain drawbacks in the model. (1) The model does not account at all for the phase response curves during the subjective day. Dead zones are not seen in the model during the subjective day. (2) The parameters in positive feedback loops are not robust to small variations. There may be other parameters which can make the model robust. But other parameter sets may not account for certain other experimental aspects. This can be solved only by knowing the parameters from the experiments. (3) Even though PER and CRY have not been considered separately, a plausible explanation for the observation of $Per2^{Brdm1}/Cry2^{-/-}$ and $Cry1^{-/-}/Cry2^{-/-}$ double mutants are given by changing the parameters of interlocked positive feedback loops in the model. However, the parameters that are changed to account for $Per2^{Brdm1}/Cry2^{-/-}$ and $Cry1^{-/-}/Cry2^{-/-}$ double mutants can be biased and this will be dealt with more elaborately at a later stage by considering PER and CRY as two separate variables.

Acknowledgements

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