

# Modeling the mammalian circadian clock: Sensitivity analysis and multiplicity of oscillatory mechanisms

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## Abstract

We extend the study of a computational model recently proposed for the mammalian circadian clock (Proc. Natl Acad. Sci. USA 100 (2003) 7051). The model, based on the intertwined positive and negative regulatory loops involving the *Per*, *Cry*, *Bmll*, and *Clock* genes, can give rise to sustained circadian oscillations in conditions of continuous darkness. These limit cycle oscillations correspond to circadian rhythms autonomously generated by suprachiasmatic nuclei and by some peripheral tissues. By using different sets of parameter values producing circadian oscillations, we compare the effect of the various parameters and show that both the occurrence and the period of the oscillations are generally most sensitive to parameters related to synthesis or degradation of *Bmll* mRNA and BMAL1 protein. The mechanism of circadian oscillations relies on the formation of an inactive complex between PER and CRY and the activators CLOCK and BMAL1 that enhance *Per* and *Cry* expression. Bifurcation diagrams and computer simulations nevertheless indicate the possible existence of a second source of oscillatory behavior. Thus, sustained oscillations might arise from the sole negative autoregulation of *Bmll* expression. This second oscillatory mechanism may not be functional in physiological conditions, and its period need not necessarily be circadian. When incorporating the light-induced expression of the *Per* gene, the model accounts for entrainment of the oscillations by light–dark (LD) cycles. Long-term suppression of circadian oscillations by a single light pulse can occur in the model when a stable steady state coexists with a stable limit cycle. The phase of the oscillations upon entrainment in LD critically depends on the parameters that govern the level of CRY protein. Small changes in the parameters governing CRY levels can shift the peak in *Per* mRNA from the L to the D phase, or can prevent entrainment. The results are discussed in relation to physiological disorders of the sleep–wake cycle linked to perturbations of the human circadian clock, such as the familial advanced sleep phase syndrome or the non-24 h sleep–wake syndrome.

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## 1. Introduction

Circadian rhythms are sustained oscillations which occur spontaneously with a period close to 24 h in most living organisms, from cyanobacteria to plants, insects and mammals. These rhythms are endogenous, as they can be observed in constant environmental conditions, e.g. continuous darkness or light. The molecular mechanism of circadian rhythms has largely been uncovered during the last decade, thanks to rapid experimental advances. While the most studied organ-

isms were initially *Drosophila* (Allada et al., 2001; Williams and Sehgal, 2001; Young and Kay, 2001) and *Neurospora* (Loros and Dunlap, 2001), molecular studies of circadian rhythms were later extended to cyanobacteria (Mori and Johnson, 2001), plants (Barak et al., 2000; Roden and Carre, 2001; Eriksson and Millar, 2003) and mammals (Allada et al., 2001; Reppert and Weaver, 2002; Okamura et al., 2002). In all cases investigated so far, the molecular mechanism of circadian oscillations relies on negative autoregulation of gene expression (Hardin et al., 1990; Glossop et al., 1999; Lee et al., 2000; Shearman et al., 2000).

In *Drosophila* (Glossop et al., 1999; Young and Kay, 2001), the PER and TIM proteins form a complex that indirectly represses the activation of the *per* and *tim*

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genes. Transcription of these genes is triggered by the complex that is formed by the activators CYC and CLOCK. Binding of the PER-TIM complex to CYC and CLOCK prevents the activation of *per* and *tim* expression. In mammals the situation resembles that observed in *Drosophila*, but it is the CRY protein that forms a regulatory complex with a PER protein (Shearman et al., 2000; Reppert and Weaver, 2002). However, a role for TIM in mammals has recently been reported (Barnes et al., 2003). The products of the *Clock* and *Bmal1* genes form the complex CLOCK-BMAL1 which activates *Per* and *Cry* transcription (Lee et al., 2001; Reppert and Weaver, 2002). The PER-CRY complex inhibits the expression of the *Per* and *Cry* genes in an indirect manner, by binding to the complex CLOCK-BMAL1. Several forms of these proteins exist (PER1, PER2, PER3, CRY1, CRY2).

The mechanism of circadian rhythms relies on interlocked negative and positive feedback loops. Besides the negative regulation of gene expression described above, indirect positive regulation is also involved. In *Drosophila*, the PER-TIM complex derepresses the transcription of *clock* by binding to CLOCK, which exerts a negative autoregulation on the expression of its gene (Bae et al., 1998) via the product of the *vri* gene (Blau and Young, 1999). In mammals, likewise, *Bmal1* expression is subjected to negative autoregulation by BMAL1, via the product of the *Rev-Erb $\alpha$*  gene (Preitner et al., 2002). The PER-CRY complex enhances *Bmal1* expression in an indirect manner (Reppert and Weaver, 2002), by binding to CLOCK-BMAL1 and thereby decreasing the transcription of the *Rev-Erb $\alpha$*  gene (Preitner et al., 2002).

The primary function of circadian rhythms is to allow biological organisms to adapt to their periodically varying environment, through entrainment of circadian rhythms by light-dark (LD) cycles. Light exerts its effects on circadian rhythms by inducing degradation of the TIM protein in *Drosophila* (Zeng et al., 1996), while in *Neurospora* (Loros and Dunlap, 2001) and mammals (Zylka et al., 1998), it acts by inducing the expression of the *Frq* and *Per* genes, respectively.

Mathematical models for circadian rhythms have been considered for long. Before data were available as to the underlying molecular mechanism, abstract physical models were used to probe properties of circadian oscillations. Thus, the Van der Pol limit cycle oscillator served as a useful model for studying entrainment of circadian rhythms by LD cycles, and phase shifts by light pulses. Art Winfree considered the synchronization of a population of circadian oscillators described in terms of their period and phase (Winfree, 1967, 1970). He also studied, both experimentally and theoretically, the classification of phase response curves

and the suppression of circadian rhythms by a critical perturbation that brings the oscillatory system back to the phase singularity, i.e. the steady state around which sustained oscillations occur (Winfree, 1973, 1980). This approach has proved highly useful as it uncovers dynamic properties of circadian rhythms, which are largely independent from the underlying molecular mechanism. The Van der Pol model is still used today, for example in the study of the response of the human circadian clock to perturbations by light pulses (Jewett et al., 1999), and in analyzing the evolutionary significance of circadian rhythms in cyanobacteria (Gonze et al., 2002b) as well as the dynamics of a population of coupled oscillators representing circadian pacemaker cells in the suprachiasmatic nuclei (Kunz and Achermann, 2003).

To account for the effects of the various genes that control circadian clocks, it becomes necessary to resort to computational models based on molecular mechanisms. Such models were first proposed for *Drosophila* (Goldbeter, 1995, 1996; Leloup and Goldbeter, 1998; Tyson et al., 1999; Ueda et al., 2001; Smolen et al., 2001, 2004; Kurosawa et al., 2002) and *Neurospora* (Leloup et al., 1999; Ruoff et al., 2001; Smolen et al., 2001). The models predict that in a certain range of parameter values the genetic regulatory network undergoes circadian oscillations of the limit cycle type, whereas outside this range the gene network operates in a stable steady state. The computational approach to circadian rhythms has recently been reviewed (Leloup and Goldbeter, 2000; Goldbeter, 2002). While these models are generally deterministic, similar results were obtained by means of stochastic simulations which show that circadian rhythms remain robust with respect to molecular noise as long as the maximum numbers of mRNA and protein molecules involved in the oscillations are in the order of tens and hundreds, respectively (Gonze et al., 2002a).

We recently proposed a deterministic model for the mammalian circadian clock (Leloup and Goldbeter, 2003). This model incorporates the regulatory effects exerted on gene expression by the PER, CRY, BMAL1, and CLOCK proteins. It also includes post-translational regulation of these proteins by reversible phosphorylation, and light-induced *Per* expression. The model accounts for sustained circadian oscillations in conditions corresponding to continuous darkness, and uncovers the possibility of multiple sources of periodic behavior in the genetic regulatory network controlling circadian oscillations. We used this model to address disorders of the sleep-wake cycle in humans, linked to perturbations of the circadian clock, such as the shift of phase observed for some parameter values upon entrainment by LD cycles, or the lack of entrainment in LD cycles (Leloup and Goldbeter, 2003).

Here, we extend the study of the model for the mammalian circadian clock by investigating the sensitivity of the predicted circadian behavior with respect to the various parameters of the system. This sensitivity analysis is performed for several sets of parameter values producing circadian oscillations. Among the parameters investigated are the rate constants for the formation and dissociation of various protein complexes, the dissociation constants measuring activation or inhibition of gene expression, and the kinetic parameters controlling the levels of mRNAs and proteins. We focus, in particular, on the parameters that govern the synthesis and degradation of *Bmall* mRNA and BMAL1 protein, to which the circadian mechanism appears to be most sensitive. We also show that the phase of circadian oscillations during entrainment in LD critically depends on the parameters that govern the level of CRY protein.

## 2. Structure of the model for the mammalian circadian clock

The model, schematized in Fig. 1, describes the regulatory interactions between the products of the *Per*, *Cry*, *Bmall*, and *Clock* genes. For simplicity, we do not distinguish between the *Per1*, *Per2*, *Per3* genes and represent them in the model by a single *Per* gene; similarly *Cry1* and *Cry2* are represented by a single *Cry* gene.

We shall treat the regulatory effect of BMAL1 on *Bmall* expression as a direct, negative autoregulation. We showed elsewhere (Leloup and Goldbeter, 2003) that similar conclusions are reached in an extended model that takes explicitly into account the action of the REV-ERB $\alpha$  protein in the indirect negative feedback exerted by BMAL1 on the expression of its gene. The version of the model without REV-ERB $\alpha$  is governed by a set of 16

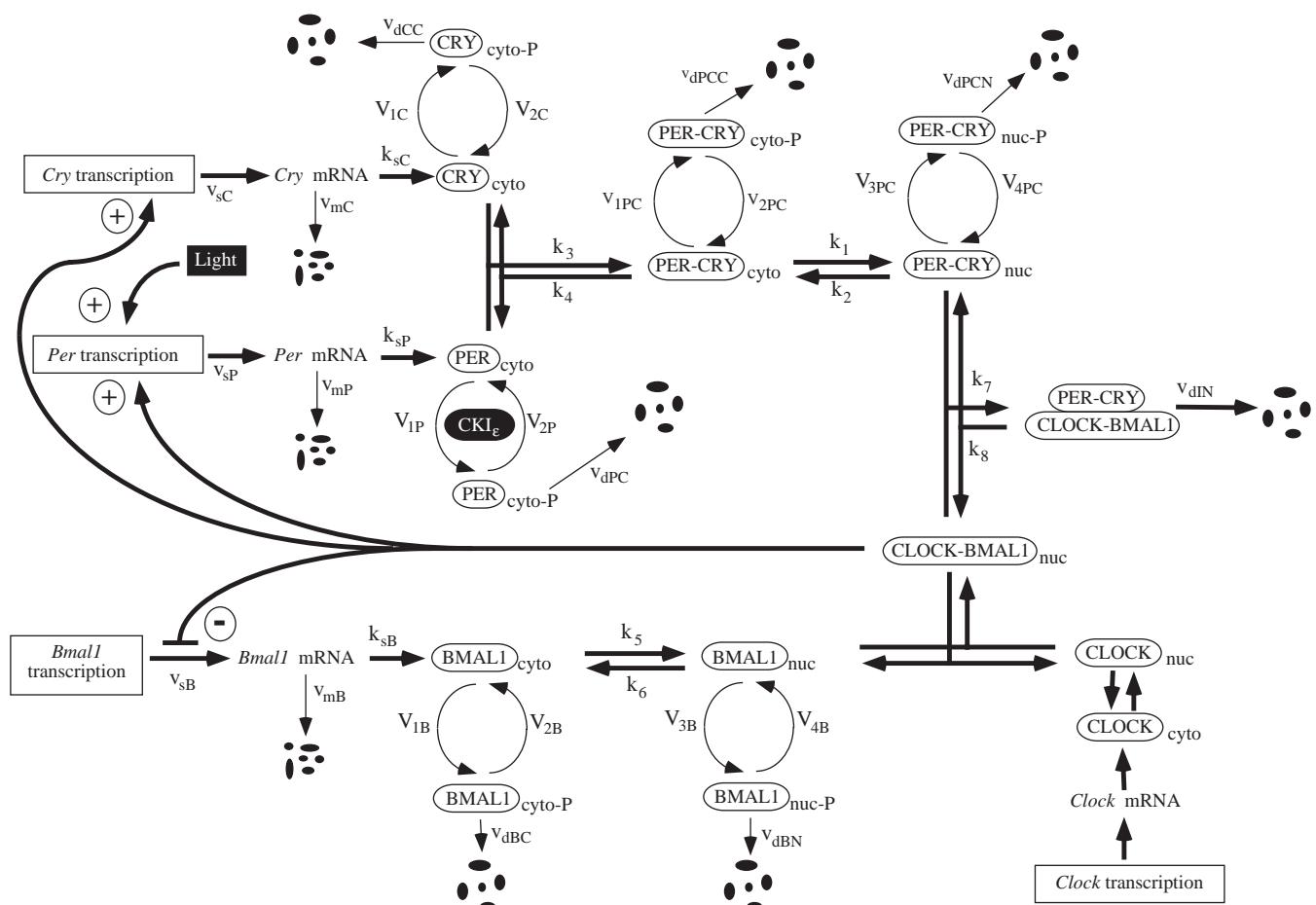


Fig. 1. Model for circadian oscillations in mammals involving interlocked negative and positive regulations of *Per*, *Cry*, *Bmall* genes by their protein products (Leloup and Goldbeter, 2003). *Per*, *Cry*, and *Bmall* mRNAs are synthesized in the nucleus and transferred into the cytosol. There they are degraded and translated into the *PER*, *CRY*, and *BMAL1* proteins, which undergo reversible phosphorylation that leads to their degradation. The complex formed by the cytosolic unphosphorylated forms of *PER* and *CRY*, and the cytosolic *BMAL1* protein are reversibly transported into the nucleus. As the expression of *Clock* is considered to be constitutive and to give rise to a high, constant level of cytosolic and nuclear *CLOCK* protein, we assume that once in the nucleus, unphosphorylated *BMAL1* immediately forms a complex with *CLOCK*. Nuclear *BMAL1* activates *Per* and *Cry* transcription and inhibits *Bmall* transcription. These regulations are counteracted by the reversible formation of an inactive complex between the nuclear, unphosphorylated *PER*-*CRY* and *CLOCK*-*BMAL1* complexes. The effect of light is to increase the rate of expression of the *Per* gene.

kinetic equations, while three more equations are needed in the model incorporating the *Rev-Erb $\alpha$*  mRNA and the REV-ERB $\alpha$  protein (Leloup and Goldbeter, 2003). A much more detailed model for the mammalian clock, containing 73 differential equations, has recently been proposed by Forger and Peskin (2003).

The model of Fig. 1 incorporates the following molecular processes (in parentheses we give the symbols denoting the concentrations of the different variables that appear in the equations listed below):

- (1) Transcription of the *Per*, *Cry* and *Bmall* genes into the corresponding mRNAs (denoted  $M_P$ ,  $M_C$ ,  $M_B$ , respectively) and degradation of these mRNAs. For simplicity, at this stage we do not distinguish between the *Per1*, *Per2*, *Per3* genes and represent them in the model by a single *Per* gene; similarly *Cry1* and *Cry2* are represented by a single *Cry* gene.
- (2) Translation of these mRNAs into the cytosolic, unphosphorylated proteins PER, CRY and BMAL1 (denoted by  $P_C$ ,  $C_C$ ,  $B_C$ ).
- (3) Reversible phosphorylation of the PER, CRY and BMAL1 proteins (concentrations of the phosphorylated forms are denoted by  $P_{CP}$ ,  $C_{CP}$ ,  $B_{CP}$ ).
- (4) In the cytosol, formation of the unphosphorylated PER–CRY complex (of concentration  $P_C$ ) and reversible phosphorylation of this complex (the concentration of the phosphorylated form is denoted by  $P_{CCP}$ ).
- (5) Reversible entry of the cytosolic PER–CRY complex into the nucleus and reversible phosphorylation of the complex (concentrations of the nuclear forms of the unphosphorylated and phosphorylated complexes are denoted by  $P_C$ <sub>N</sub> and  $P_{NP}$ , respectively).
- (6) Reversible entry of the cytosolic BMAL1 protein into the nucleus and reversible phosphorylation (concentrations of the nuclear forms of unphosphorylated and phosphorylated BMAL1 are denoted by  $B_N$  and  $B_{NP}$ , respectively).
- (7) In agreement with experimental observations, the expression of *Clock* is considered to be constitutive and to give rise to a high, constant level of cytosolic and nuclear CLOCK protein (Reppert and Weaver, 2001). We will not distinguish between the phosphorylated and unphosphorylated forms of CLOCK and will treat its constant level as a parameter. We assume that once in the nucleus, unphosphorylated BMAL1 immediately forms a complex with CLOCK (the concentration of this complex is that of nuclear BMAL1, i.e.  $B_N$ ).
- (8) In the nucleus, the CLOCK–BMAL1 activates the transcription of the *Per* and *Cry* genes. By binding to the CLOCK–BMAL1 complex, the PER–CRY complex prevents this activation; such a regulation therefore amounts to indirect repression of the *Per*

and *Cry* genes by their protein products (the concentration of the inactive complex between CLOCK–BMAL1 and PER–CRY is denoted  $I_N$ ).

- (9) Experimental evidence indicates that PER2, and to a lesser degree CRY1 and CRY2, behave as activators of *Bmall* transcription (Reppert and Weaver, 2001; Yu et al., 2002). However, the precise mechanism of this regulation is not yet fully clarified. In analogy with the situation in *Drosophila*, we assume that the positive feedback occurs indirectly and that CLOCK–BMAL1 represses the transcription of the *Bmall* gene; the activating effect of PER2, CRY1 and CRY2 would be due to the removal of repression upon formation of the complex between PER–CRY and CLOCK–BMAL1.
- (10) The negative autoregulation exerted by BMAL1 on the expression of its gene was recently shown to be of indirect nature: BMAL1 promotes the expression of the *Rev-Erb $\alpha$*  gene and the REV-ERB $\alpha$  protein represses the expression of *Bmall* (Preitner et al., 2002). We shall consider the regulatory effect of BMAL1 as a direct, negative autoregulation. As shown in our previous publication (Leloup and Goldbeter, 2003) similar results are obtained when the action of REV-ERB $\alpha$  in the regulation of *Bmall* expression is considered explicitly.
- (11) Although the proteins may be multiply phosphorylated (Lee et al., 2001), we will only consider a single phosphorylated state for PER, CRY, BMAL1 and the complex PER–CRY. We assume that these phosphorylated proteins are subject to degradation in the cytosol and in the nucleus. Degradation is also considered for the nuclear, unphosphorylated form of the complex  $I_N$  between PER–CRY and CLOCK–BMAL1; the introduction of a phosphorylation step prior to degradation of  $I_N$  would introduce an additional variable but does not change significantly the behavior of the model.
- (12) The present work deals with the dynamics of the model in conditions corresponding to continuous darkness or to light–dark cycles. The effect of light is to enhance transcription of the *Per* gene and is therefore incorporated into the model through the maximum rate of *Per* expression, denoted by  $v_{SP}$  in the model.

A family of closely related models can be built, based on the above assumptions. We shall focus here on one particular implementation of this family of models. Alternative versions of the circadian clock model yield largely similar results. Thus, BMAL1 may form a complex with CLOCK before entering the nucleus, and complexes between CRY and PER or between CLOCK and BMAL1 may form when the various proteins are phosphorylated (Lee et al., 2001).

### 3. Kinetic equations

The time evolution of the model of Fig. 1 is governed by the system of 16 kinetic Eqs. (1)–(16). For the sake of clarity, we have grouped these equations for the various mRNAs, the phosphorylated and non-phosphorylated proteins PER and CRY in the cytosol, the phosphorylated and non-phosphorylated PER–CRY complex in cytosol and nucleus, the phosphorylated and nonphosphorylated protein BMAL1 in the cytosol and nucleus, and the complex between PER–CRY and CLOCK–BMAL1 in the nucleus:

(a) mRNAs of *Per*, *Cry* and *Bmall*:

$$\frac{dM_P}{dt} = v_{sP} \frac{B_N^n}{K_{AP}^n + B_N^n} - v_{mP} \frac{M_P}{K_{mP} + M_P} - k_{dmp} M_P, \quad (1)$$

$$\frac{dM_C}{dt} = v_{sC} \frac{B_N^n}{K_{AC}^n + B_N^n} - v_{mC} \frac{M_C}{K_{mC} + M_C} - k_{dmc} M_C, \quad (2)$$

$$\frac{dM_B}{dt} = v_{sB} \frac{K_{IB}^m}{K_{IB}^m + B_N^m} - v_{mB} \frac{M_B}{K_{mB} + M_B} - k_{dmB} M_B. \quad (3)$$

(b) Phosphorylated and non-phosphorylated proteins PER and CRY in the cytosol:

$$\begin{aligned} \frac{dP_C}{dt} = & k_{sP} M_P - V_{1P} \frac{P_C}{K_p + P_C} + V_{2P} \frac{P_{CP}}{K_{dp} + P_{CP}} \\ & + k_4 P_{CC} - k_3 P_C C_C - k_{dn} P_C, \end{aligned} \quad (4)$$

$$\begin{aligned} \frac{dC_C}{dt} = & k_{sC} M_C - V_{1C} \frac{C_C}{K_p + C_C} + V_{2C} \frac{C_{CP}}{K_{dp} + C_{CP}} \\ & + k_4 P_{CC} - k_3 P_C C_C - k_{dn} C_C, \end{aligned} \quad (5)$$

$$\begin{aligned} \frac{dP_{CP}}{dt} = & V_{1P} \frac{P_C}{K_p + P_C} - V_{2P} \frac{P_{CP}}{K_{dp} + P_{CP}} \\ & - v_{dPC} \frac{P_{CP}}{K_d + P_{CP}} - k_{dn} P_{CP}, \end{aligned} \quad (6)$$

$$\begin{aligned} \frac{dC_{CP}}{dt} = & V_{1C} \frac{C_C}{K_p + C_C} - V_{2C} \frac{C_{CP}}{K_{dp} + C_{CP}} \\ & - v_{dCC} \frac{C_{CP}}{K_d + C_{CP}} - k_{dn} C_{CP}. \end{aligned} \quad (7)$$

(c) Phosphorylated and non-phosphorylated PER–CRY complex in cytosol and nucleus:

$$\begin{aligned} \frac{dP_C}{dt} = & -V_{1PC} \frac{P_C}{K_p + P_C} + V_{2PC} \frac{P_{CP}}{K_{dp} + P_{CP}} \\ & - k_4 P_{CC} + k_3 P_C C_C + k_2 P_C N - k_1 P_C C - k_{dn} P_C, \end{aligned} \quad (8)$$

$$\begin{aligned} \frac{dP_C}{dt} = & -V_{3PC} \frac{P_C}{K_p + P_C} + V_{4PC} \frac{P_{NP}}{K_{dp} + P_{NP}} \\ & - k_2 P_C N + k_1 P_C C - k_7 B_N P_C N + k_8 I_N - k_{dn} P_C N, \end{aligned} \quad (9)$$

$$\begin{aligned} \frac{dP_{CP}}{dt} = & V_{1PC} \frac{P_C}{K_p + P_C} - V_{2PC} \frac{P_{CP}}{K_{dp} + P_{CP}} \\ & - v_{dPC} \frac{P_{CP}}{K_d + P_{CP}} - k_{dn} P_{CP}, \end{aligned} \quad (10)$$

$$\begin{aligned} \frac{dP_{NP}}{dt} = & V_{3PC} \frac{P_C}{K_p + P_C} - V_{4PC} \frac{P_{NP}}{K_{dp} + P_{NP}} \\ & - v_{dPCN} \frac{P_{NP}}{K_d + P_{NP}} - k_{dn} P_{NP}, \end{aligned} \quad (11)$$

(d) Phosphorylated and non-phosphorylated protein BMAL1 in the cytosol and nucleus:

$$\begin{aligned} \frac{dB_C}{dt} = & k_{sB} M_B - V_{1B} \frac{B_C}{K_p + B_C} + V_{2B} \frac{B_{CP}}{K_{dp} + B_{CP}} \\ & - k_5 B_C + k_6 B_N - k_{dn} B_C, \end{aligned} \quad (12)$$

$$\begin{aligned} \frac{dB_{CP}}{dt} = & V_{1B} \frac{B_C}{K_p + B_C} - V_{2B} \frac{B_{CP}}{K_{dp} + B_{CP}} \\ & - v_{dBC} \frac{B_{CP}}{K_d + B_{CP}} - k_{dn} B_{CP}, \end{aligned} \quad (13)$$

$$\begin{aligned} \frac{dB_N}{dt} = & -V_{3B} \frac{B_N}{K_p + B_N} + V_{4B} \frac{B_{NP}}{K_{dp} + B_{NP}} \\ & + k_5 B_C - k_6 B_N - k_7 B_N P_C N + k_8 I_N - k_{dn} B_N, \end{aligned} \quad (14)$$

$$\begin{aligned} \frac{dB_{NP}}{dt} = & V_{3B} \frac{B_N}{K_p + B_N} - V_{4B} \frac{B_{NP}}{K_{dp} + B_{NP}} \\ & - v_{dBN} \frac{B_{NP}}{K_d + B_{NP}} - k_{dn} B_{NP}. \end{aligned} \quad (15)$$

(e) Inactive complex between PER–CRY and CLOCK–BMAL1 in nucleus:

$$\begin{aligned} \frac{dI_N}{dt} = & -k_8 I_N + k_7 B_N P_C N \\ & - v_{dIN} \frac{I_N}{K_d + I_N} - k_{dn} I_N. \end{aligned} \quad (16)$$

The definition of the various parameters is indicated in Table 1. In Eqs. (1)–(16), concentrations are defined with respect to the total cell volume. The concentration of every protein species (single protein or complex between two or more proteins) is denoted by a subscript *C*, *N*, *CP* or *NP* for cytosolic, nuclear, cytosolic phosphorylated or nuclear phosphorylated, respectively. Thus, an expression such as *P<sub>C</sub>* refers to the concentration of the cytosolic complex between PER

Table 1

Four different sets of basal parameter values yielding circadian oscillations in conditions corresponding to continuous darkness

Parameter	Definition	Set 1	Set 2	Set 3	Set 4
$k_1 (\text{h}^{-1})$	Rate constant for entry of the PER–CRY complex into the nucleus	0.4	0.3	0.2	0.8
$k_2 (\text{h}^{-1})$	Rate constant for exit of the PER–CRY complex from the nucleus	0.2	0.3	0.3	0.2
$k_3 (\text{nM}^{-1} \text{h}^{-1})$	Rate constant for the formation of the PER–CRY complex	0.4	0.7	0.4	0.8
$k_4 (\text{h}^{-1})$	Rate constant for dissociation of the PER–CRY complex	0.2	0.7	0.3	0.2
$k_5 (\text{h}^{-1})$	Rate constant for entry of the BMAL1 protein into the nucleus	0.4	0.4	0.4	0.4
$k_6 (\text{h}^{-1})$	Rate constant for exit of the BMAL1 protein from the nucleus	0.2	0.4	0.3	0.2
$k_7 (\text{nM}^{-1} \text{h}^{-1})$	Rate constant for the formation of the inactive PER–CRY–CLOCK–BMAL1 complex	0.5	0.8	0.6	0.5
$k_8 (\text{h}^{-1})$	Rate constant for the dissociation of the PER–CRY–CLOCK–BMAL1 complex	0.1	0.6	0.4	0.1
$K_{AP} (\text{nM})$	Activation constant for enhancement of <i>Per</i> expression by nuclear BMAL1	0.7	1.0	1.0	0.7
$K_{AC} (\text{nM})$	Activation constant for enhancement of <i>Cry</i> expression by nuclear BMAL1	0.6	1.0	1.0	1.0
$K_{IB} (\text{nM})$	Inhibition constant for repression of <i>Bmll</i> expression by nuclear BMAL1	2.2	1.0	2.5	0.8
$k_{dmb} (\text{h}^{-1})$	Nonspecific degradation rate constant for <i>Bmll</i> mRNA	0.01	0.01	0.01	0.01
$k_{dmc} (\text{h}^{-1})$	Nonspecific degradation rate constant for <i>Cry</i> mRNA	0.01	0.01	0.01	0.01
$k_{dmn} (\text{h}^{-1})$	Nonspecific degradation rate constant for <i>Per</i> mRNA	0.01	0.01	0.01	0.01
$k_{dnc} (\text{h}^{-1})$	Nonspecific degradation rate constant for cytosolic non-phosphorylated CRY	0.12	0.01	0.01	0.01
$k_{dn} (\text{h}^{-1})$	Nonspecific degradation rate constant for other protein species	0.01	0.01	0.01	0.01
$K_d (\text{nM})$	Michaelis constant for protein degradation	0.3	0.7	0.7	0.3
$K_{dp} (\text{nM})$	Michaelis constant for protein dephosphorylation	0.1	0.6	0.6	0.3
$K_p (\text{nM})$	Michaelis constant for protein phosphorylation	0.1	0.2	0.5	0.1
$K_{mB} (\text{nM})$	Michaelis constant for degradation of <i>Bmll</i> mRNA	0.4	1.0	1.0	0.4
$K_{mC} (\text{nM})$	Michaelis constant for degradation of <i>Cry</i> mRNA	0.4	0.7	0.9	0.4
$K_{mP} (\text{nM})$	Michaelis constant for degradation of <i>Per</i> mRNA	0.31	1.0	0.7	0.3
$k_{stot} (\text{h}^{-1})$	Rate constant for protein synthesis	1.0	1.0	1.0	1.0
$k_{sB} (\text{h}^{-1})$	Rate constant for synthesis of BMAL1	0.12 $k_{stot}$	0.4 $k_{stot}$	0.2 $k_{stot}$	$k_{stot}$
$k_{sC} (\text{h}^{-1})$	Rate constant for synthesis of CRY	1.6 $k_{stot}$	0.8 $k_{stot}$	0.8 $k_{stot}$	$k_{stot}$
$k_{sP} (\text{h}^{-1})$	Rate constant for synthesis of PER	0.6 $k_{stot}$	0.65 $k_{stot}$	2.0 $k_{stot}$	0.5 $k_{stot}$
$n$	Degree of cooperativity of activation of <i>Per</i> and <i>Cry</i> expression by BMAL1	4	4	4	4
$m$	Degree of cooperativity of repression of <i>Bmll</i> expression by BMAL1	2	4	4	4
$V_{phos} (\text{nMh}^{-1})$	Phosphorylation rate	0.4	1.0	1.0	0.6
$V_{1B} (\text{nMh}^{-1})$	Maximum rate of cytosolic BMAL1 phosphorylation	0.5	1.0	0.6	1.0
$V_{1C} (\text{nMh}^{-1})$	Maximum rate of cytosolic CRY phosphorylation	0.6	0.3	0.6	0.6
$V_{1P} (\text{nMh}^{-1})$	Maximum rate of cytosolic PER phosphorylation	$V_{phos}$	$V_{phos}$	$V_{phos}$	$V_{phos}$
$V_{1PC} (\text{nMh}^{-1})$	Maximum rate of phosphorylation of cytosolic PER–CRY complex	$V_{phos}$	1.2 $V_{phos}$	1.2 $V_{phos}$	$V_{phos}$
$V_{2B} (\text{nMh}^{-1})$	Maximum rate of cytosolic BMAL1 dephosphorylation	0.1	0.3	0.3	0.1
$V_{2C} (\text{nMh}^{-1})$	Maximum rate of cytosolic CRY dephosphorylation	0.1	0.1	0.2	0.1
$V_{2P} (\text{nMh}^{-1})$	Maximum rate of cytosolic PER dephosphorylation	0.3	0.3	0.3	0.3
$V_{2PC} (\text{nMh}^{-1})$	Maximum rate of cytosolic PER–CRY complex dephosphorylation	0.1	0.5	0.5	0.1
$V_{3B} (\text{nMh}^{-1})$	Maximum rate of nuclear BMAL1 phosphorylation	0.5	1.0	1.0	1.0
$V_{3PC} (\text{nMh}^{-1})$	Maximum rate of phosphorylation of nuclear PER–CRY complex	$V_{phos}$	1.2 $V_{phos}$	1.2 $V_{phos}$	$V_{phos}$
$V_{4B} (\text{nMh}^{-1})$	Maximum rate of nuclear BMAL1 dephosphorylation	0.2	0.3	0.3	0.2
$V_{4PC} (\text{nMh}^{-1})$	Maximum rate of dephosphorylation of nuclear PER–CRY complex	0.1	0.5	0.3	0.1
$v_{dbc} (\text{nMh}^{-1})$	Maximum rate of degradation of cytosolic phosphorylated BMAL1	0.5	1.4	1.4	1.0
$v_{dbN} (\text{nMh}^{-1})$	Maximum rate of degradation of nuclear phosphorylated BMAL1	0.6	0.2	0.2	0.5
$v_{dCC} (\text{nMh}^{-1})$	Maximum rate of degradation of cytosolic phosphorylated CRY	0.7	0.5	0.5	0.7
$v_{dIN} (\text{nMh}^{-1})$	Maximum rate of degradation of nuclear PER–CRY–CLOCK–BMAL1 complex	0.8	1.2	1.2	0.8
$v_{dPC} (\text{nMh}^{-1})$	Maximum rate of degradation of cytosolic phosphorylated PER	0.7	0.8	0.8	0.7
$v_{dpcc} (\text{nMh}^{-1})$	Maximum rate of degradation of cytosolic phosphorylated PER–CRY complex	0.7	0.3	0.3	1.0
$v_{dpCN} (\text{nMh}^{-1})$	Maximum rate of degradation of nuclear phosphorylated PER–CRY complex	0.7	0.3	0.3	1.0
$v_{mB} (\text{nMh}^{-1})$	Maximum rate of <i>Bmll</i> mRNA degradation	0.8	0.5	0.5	0.2
$v_{mC} (\text{nMh}^{-1})$	Maximum rate of <i>Cry</i> mRNA degradation	1.0	0.6	0.8	1.0
$v_{mP} (\text{nMh}^{-1})$	Maximum rate of <i>Per</i> mRNA degradation	1.1	1.0	0.7	1.1
$v_{sTot} (\text{nMh}^{-1})$	Maximum transcription rate	1.0	1.0	1.0	1.0
$v_{sB} (\text{nMh}^{-1})$	Maximum rate of <i>Bmll</i> mRNA synthesis	$v_{stot}$	1.2 $v_{stot}$	0.5 $v_{stot}$	0.7 $v_{stot}$
$v_{sC} (\text{nMh}^{-1})$	Maximum rate of <i>Cry</i> mRNA synthesis	1.1 $v_{stot}$	1.5 $v_{stot}$	1.5 $v_{stot}$	0.8 $v_{stot}$
$v_{sP} (\text{nMh}^{-1})$	Maximum rate of <i>Per</i> mRNA synthesis	1.5 $v_{stot}$	1.3 $v_{stot}$	1.1 $v_{stot}$	$v_{stot}$

For sets 1, 2, 3 and 4, the autonomous period is 23.8 h, 24.1 h, 23.8 h and 24.2 h, respectively. Parameter set 1 is the one used in our previous publication (Leloup and Goldbeter, 2003): oscillations disappear when PER is absent, or when the negative autoregulation by BMAL1 is not operative. Parameter set 2 corresponds to a situation in which oscillations can occur in the absence of PER ( $k_{sP} = 0$ ), as a result of the negative autoregulation of BMAL1 (see Fig. 3B). Parameter set 3 corresponds to a situation in which circadian oscillations can occur in the absence of the negative autoregulation of BMAL1 ( $K_{IB} \geq 100$ ). Finally, parameter set 4 was selected because oscillations can occur either in the absence of PER or in the absence of negative autoregulation by BMAL1. In the latter case, oscillations are less sensitive to parameters controlling levels of BMAL1 and its mRNA.

and CRY, while the product of the concentrations of PER and CRY in the cytosol is denoted  $P_C C_C$ .

#### 4. Endogenous circadian oscillations

We previously showed (Leloup and Goldbeter, 2003) that in a certain range of parameter values the system of Eqs. (1)–(16) produces sustained oscillations with a circadian period. These oscillations are endogenous, since they occur for parameter values that remain constant in time, in agreement with the observation that circadian rhythms in mammals persist in continuous darkness or light. As observed experimentally (Lee et al., 2001; Reppert and Weaver, 2002), *Bmal1* mRNA oscillates in antiphase with *Per* and *Cry* mRNAs (Fig. 2A). The proteins undergo similar oscillations and follow their mRNA by a few hours (Fig. 2B). Because most parameter values remain to be determined experimentally, these oscillations were obtained for a semi-arbitrary choice of parameter values, in a physiological range, so as to yield a period of oscillations in continuous darkness (DD) close to 24 h.

Sustained oscillations correspond to the rhythms produced by suprachiasmatic nuclei (SCN) located in the hypothalamus, which behave as the circadian pacemaker in mammals (Reppert and Weaver, 2002; Sujino et al., 2003). The oscillations also correspond to circadian rhythms generated by the same genetic control network in peripheral mammalian tissues such as liver, heart, kidney, and pancreas (Balsalobre et al., 1998; Schibler et al., 2003). These peripheral circadian rhythms also appear to be sustained (Yoo et al., 2004).

To investigate properties of the oscillations predicted by the model we considered seven different sets of parameter values producing endogenous circadian oscillations. Parameter values for sets numbered 1–4, which correspond to significantly different conditions, are listed in Table 1. The oscillations shown in panels A, B and C, D in Fig. 2 were obtained for parameter sets 1 and 4, respectively. Sustained oscillations occur in an appropriate window of parameter values. Outside this range, the system evolves toward a stable steady state. Such an evolution is often accompanied by damped oscillations.

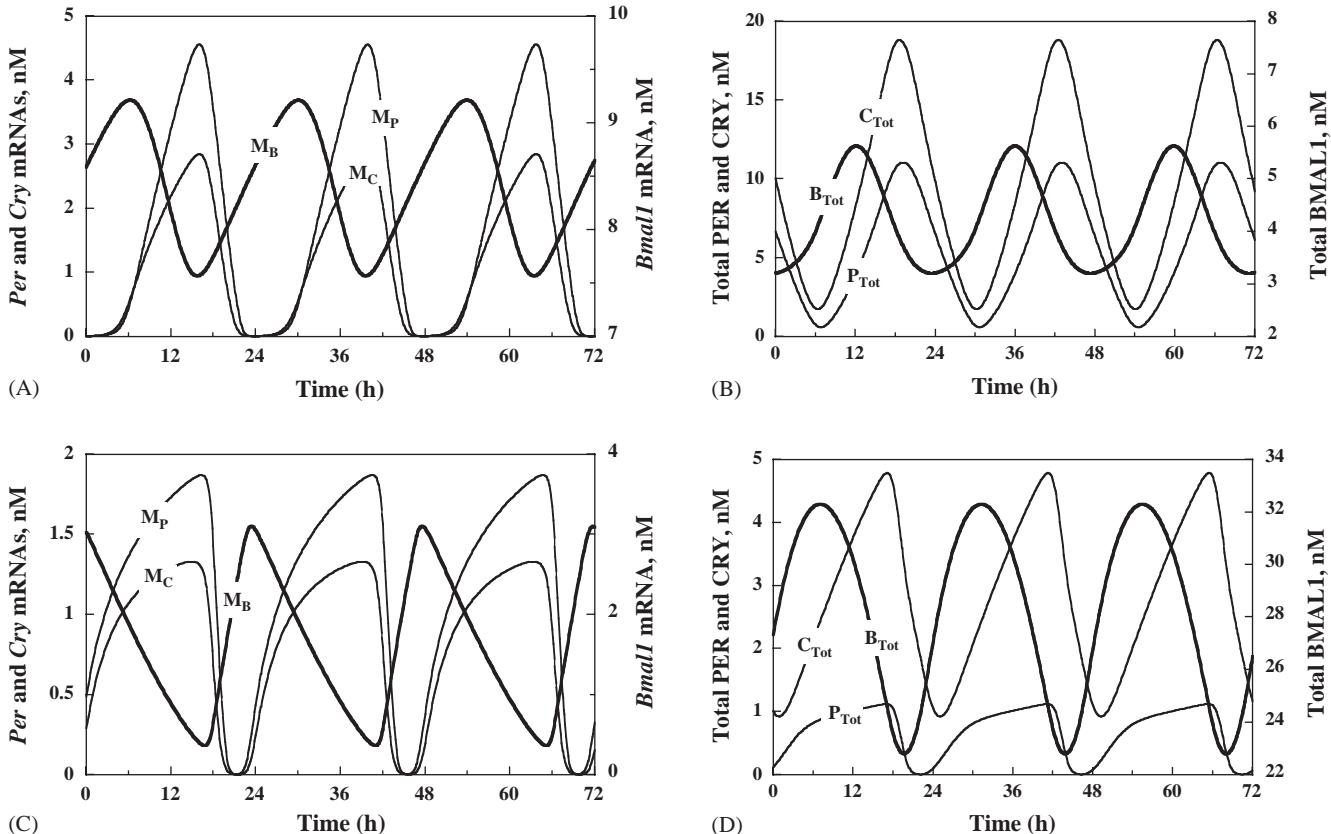


Fig. 2. Circadian oscillations in constant darkness. (A, C) Time evolution of the mRNAs of *Per* ( $M_P$ ), *Cry* ( $M_C$ ) and *Bmal1* ( $M_B$ ). (B, D) Corresponding oscillations of the total amounts of PER ( $P_{Tot}$ ), CRY ( $C_{Tot}$ ) and BMAL1 ( $B_{Tot}$ ) proteins. The curves have been obtained by numerical integration of Eqs. (1)–(16) for parameter set 1 in (A, B) and parameter set 4 in (C, D). For both parameter sets the *Bmal1* mRNA oscillates in antiphase with respect to the mRNAs of *Per* and *Cry*.

## 5. Identification of a second oscillatory mechanism based on negative autoregulation of BMAL1

The genetic regulatory network underlying circadian rhythms contains intertwined positive and negative feedback loops (see Fig. 1). In view of the complexity of these regulatory interactions, the possibility arises that more than one mechanism in the network may give rise to sustained oscillations. We previously obtained evidence for a second oscillatory mechanism by observing that sustained oscillations may occur even in the absence of PER protein (Leloup and Goldbeter, 2003). This second oscillator is based on the negative autoregulation exerted by BMAL1 on the expression of its gene, via the *Rev-Erb $\alpha$*  gene.

Experimental observations so far appear to indicate that, if a second oscillator mechanism exists in the circadian regulatory network, it does not manifest itself in producing rhythmic behavior. Thus, *mPer1/mPer2* (Zheng et al., 2001) or *mCry1/mCry2* (Van der Horst et al., 1999) double-knockout mice are arrhythmic. In some conditions, however, an extended light pulse can restore rhythmic behavior in a low proportion of *mPer1/mPer2* double-knockout mice (K. Bae and D. Weaver, pers. comm.).

In the model, we observe that in the absence of PER, depending on parameter values, the second oscillator may either be silent (Fig. 3A, established for parameter set 1) or produce by itself oscillations (Fig. 3B, established for parameter set 2). Thus, upon setting to zero the rate of synthesis of the PER protein ( $k_{SP} = 0$ ) and thereby silencing the negative feedback loop involving the PER–CRY complex, oscillations either disappear as the system evolves toward a stable steady state (Fig. 3A), or the rhythm continues, with another period (which may not necessarily be circadian) and another amplitude, dictated by the mechanism of the BMAL1 oscillator (Fig. 3B).

In the absence of the negative feedback exerted by BMAL1 on the expression of its gene, oscillations can still originate from the PER–CRY negative feedback loop involving BMAL1. This result holds with the observation that circadian oscillations occur in the absence of REV-ERB $\alpha$  in mice (Preitner et al., 2002). Preventing altogether the synthesis of BMAL1 suppresses oscillations, because BMAL1 is involved in the mechanism of the two oscillators described above.

## 6. Two types of parameter sensitivity

One way to assess the sensitivity of circadian oscillatory behavior to changes in parameter values is to determine, for each parameter, one at a time, the range of values producing sustained oscillations as well as the variation of the period over this range, while

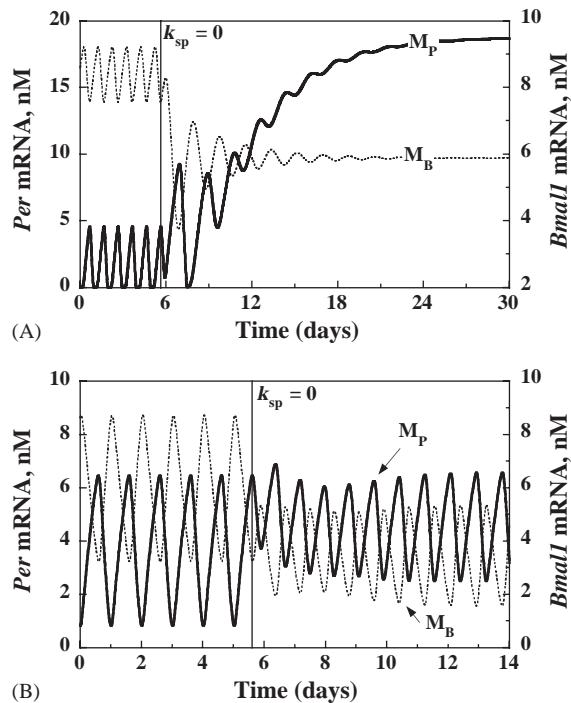


Fig. 3. Possibility of oscillations due to multiple oscillatory mechanisms. (A) The oscillations disappear in the absence of PER protein synthesis ( $k_{SP} = 0$ ). The curves show the asymptotic, stable steady state reached after transients have subsided. (B) When  $k_{SP} = 0$  sustained oscillations can however occur for other sets of parameter values, such as for set 2. The fact that oscillations can occur in the absence of PER protein indicates the existence of another oscillatory mechanism that might rely only on CLOCK–BMAL1 autoregulation. The curves, showing the time evolution of the mRNAs of *Per* ( $M_P$ ) and *Bmall* ( $M_B$ ), are obtained by numerical integration of Eqs. (1)–(16) for parameter set 1 (panel A) and parameter set 2 (panel B).

keeping for the other parameters the basal values listed in Table 1 in the appendix. Such a sensitivity analysis was previously performed for the parameter values listed as set 1 in Table 1, used in Fig. 2A and B. Here, we determine the sensitivity to the various parameters defined in Table 1 for three additional sets of parameter values (listed in Table 1) yielding circadian oscillations, and compare in Table 2 the results with those obtained for set 1. The purpose of considering different sets of parameter values is to check the generality of the conclusions reached by means of sensitivity analysis.

Parameter set 1 is that used in our previous publication (Leloup and Goldbeter, 2003): oscillations disappear in the absence of PER ( $k_{SP} = 0$ ) or in the absence of negative autoregulation by BMAL1 ( $K_{IB} \geq 100$ ). Parameter set 2 corresponds to a situation in which oscillations can occur in the absence of PER, as a result of the negative autoregulation of BMAL1 (see Fig. 3B). Parameter set 3 corresponds to a situation in which circadian oscillations can occur in the absence of negative autoregulation by BMAL1. Finally, parameter set 4 was selected because oscillations can occur in the

absence of PER or in the absence of negative autoregulation of BMAL1; moreover, in this case, oscillations are less sensitive to parameters controlling the level of BMAL1.

Two types of sensitivity are noticeable from the data in Table 2; the first relates to the size of the oscillatory domain, and the other, to the influence of each parameter on the period. For some parameters the range of values producing sustained oscillations is quite narrow, less than one order of magnitude, while for other parameters it extends over several orders of magnitude. When the window for oscillations is relatively narrow, which indicates higher sensitivity, the lower and upper bounds of the oscillatory domain in Table 2 are indicated in bold type (this is the case, for example, for parameter  $v_{mB}$  for each of the 4 sets of parameter values, relative to  $v_{mC}$  and  $v_{mP}$ ).

In regard to the second type of sensitivity, the largest variation in period, by a factor 4–6 is obtained for  $k_5, K_{IB}, v_{mB}, v_{sB}$  with parameter set 4. For the other parameter sets, the highest variations, by a factor close to 3, is observed for parameters  $k_1$  and  $k_7$  for parameter set 1. The change in period over the oscillatory range of a parameter, from one boundary to the other of the oscillatory domain, is generally less than a factor of 3. Parameters for which the variation in period is largest over the oscillatory domain are represented in bold type in Table 2; the values of the period at the borders of the oscillatory domain are also indicated in bold type for these parameters.

For some sets of parameter values, the period may vary significantly, by a factor close to 2, over the oscillatory domain while for other sets of parameter values the change in period as a function of this parameter may be reduced. This is illustrated in Table 2 by the case of parameter  $k_2$ , for which the period varies by a factor close to 2 for parameter set 2, while the corresponding variation is much smaller for parameter sets 1, 3 and 4.

## 7. The clock mechanism can be highly sensitive to BMAL1

Parameters for which the range of values producing oscillations is narrowest are mainly those linked to synthesis and degradation of BMAL1 and its mRNA ( $v_{sB}, v_{mB}, k_{sB}, K_{IB}$ , and to a lesser degree  $V_{1B}$  and  $V_{3B}$ ). This observation holds for 3 out of 4 sets of parameter values considered in Table 2. The sensitivity extends to the influence exerted by these parameters on the period. We found, however, that the enhanced sensitivity toward parameters governing the level of BMAL1 and its mRNA is not observed in all cases, as illustrated in Table 2 for parameter set 4.

A few other parameters, such as  $k_1$  and  $k_7$ , which produce oscillations over a large range of values, can change the period by a factor of about 3. Parameters related to *Bmall* mRNA, such as  $v_{sB}, v_{mB}, K_{IB}, K_{mB}$ , change the period by a smaller factor, but even though their oscillatory range is smaller they influence the period more than the corresponding parameters for *Per* or *Cry* mRNAs. The data in Table 2 nevertheless indicate that the magnitude of the changes in period due to a variation in these parameters depends on the set of parameter values considered.

On the basis of the data presented in Table 2, we may conclude, in summary, that parameters related to synthesis and degradation of BMAL1 and its mRNA possess the narrowest range of values producing sustained oscillations. For parameter set 1, the period is most affected by the parameters measuring the entry of the PER–CRY complex into the nucleus ( $k_1$ ) and the formation of the inactive complex between PER–CRY and CLOCK–BMAL1 ( $k_7$ ), while for parameter set 4, the largest effect on the period is exerted by  $k_5, K_{IB}, v_{mB}$  and  $v_{sB}$ . Thus, for parameter set 4, even if the oscillatory domains for parameters controlling BMAL1 and its mRNA are not as narrow as for parameter sets 1–3, these parameters nevertheless continue to exert a marked effect on the period.

## 8. Bifurcation diagrams for circadian oscillations

While Table 2 lists the value of the period only at the boundaries of the domain of sustained oscillations for each control parameter, a more comprehensive picture of the effect of a parameter is provided by bifurcation diagrams, which show how the period varies over the whole oscillatory range. Such bifurcation diagrams are presented for a selected choice of parameters in Figs. 4–8. Each column refers to a given parameter, and the four rows refer to the 4 sets of parameter values considered (see Table 1). We show successively the effect of: the activation ( $K_{AP}, K_{AC}$ ) and inhibition ( $K_{IB}$ ) constants (Fig. 4), some rate constants ( $k_1, k_7$ ) and the degrees of cooperativity  $m$  and  $n$  (Fig. 5), the rates of gene transcription  $v_{sP}, v_{sC}$ , and  $v_{sB}$  (Fig. 6), the rates of protein synthesis  $k_{sP}, k_{sC}$ , and  $k_{sB}$  (Fig. 7), and the global rates of gene expression  $v_{stot}$ , of protein synthesis  $k_{stot}$ , and of phosphorylation of the various forms of the PER protein,  $V_{phos}$  (Fig. 8). Thus the diagrams in Figs. 6 and 7 indicate how the period varies when the synthesis of a particular mRNA or protein is inhibited or enhanced, while the diagrams in Fig. 8 show the effect of a change in these synthesis rates for all mRNAs or all proteins involved in the circadian oscillatory mechanism.

The bifurcation diagrams indicate that the period may change monotonously as a function of a parameter or,

Table 2

Sensitivity analysis showing for each parameter of the model the range of values producing sustained oscillations and the parameter effect on the period

Parameters	Set 1		Set 2		Set 3		Set 4	
	Lower boundary	Upper boundary	Lower boundary	Upper boundary	Lower boundary	Upper boundary	Lower boundary	Upper boundary
$k_1 (\text{h}^{-1})$	0.008 ( <b>46.4 h</b> )	> 100 ( <b>14.8 h</b> )	0 (20.5 h)	4 (13.5 h)	0.05 ( <b>35 h</b> )	1.4 ( <b>14.5 h</b> )	0 (24.7 h)	> 100 (22.3 h)
$k_2 (\text{h}^{-1})$	0 (25.3 h)	3.8 (30.3 h)	0 (35 h)	1.9 (17 h)	0 (23 h)	1.1 (26 h)	0 (24.1 h)	> 100 (24.3 h)
$k_3 (\text{nM}^{-1}\text{h}^{-1})$	0.000015 ( <b>41.2 h</b> )	> 100 ( <b>22.3 h</b> )	0 (20.5 h)	> 100 (24.1 h)	0.00006 ( <b>56 h</b> )	> 100 ( <b>22.9 h</b> )	0 (24.7 h)	> 100 (23.4 h)
$k_4 (\text{h}^{-1})$	0 (24.2 h)	> 100 (27.3 h)	0 (24.5 h)	> 100 (22.5 h)	0 (23.5 h)	> 100 (32 h)	0 (24.2 h)	> 100 (24.5 h)
$k_5 (\text{h}^{-1})$	0.008 (17.7 h)	> 100 (23 h)	0.04 (20.2 h)	1.6 (13.5 h)	0.1 (21.7 h)	> 100 (25.5 h)	0.005 ( <b>67.5 h</b> )	2.1 ( <b>10.7 h</b> )
$k_6 (\text{h}^{-1})$	0 (23.7 h)	8.8 (38 h)	0 (26.2 h)	2.6 (22.7 h)	0 (23 h)	2.2 (29.8 h)	0 (25.3 h)	1.3 (19.4 h)
$k_7 (\text{nM}^{-1}\text{h}^{-1})$	0.03 ( <b>44 h</b> )	> 100 ( <b>14.7 h</b> )	0 (20.5 h)	> 100 (17.3 h)	0.07 ( <b>42 h</b> )	> 100 ( <b>15.5 h</b> )	0 (24.7 h)	> 100 (22.7 h)
$k_8 (\text{h}^{-1})$	0 (23.4 h)	11.7 (39.5 h)	0 (22.5 h)	24.1 (17.8 h)	0 ( <b>22.5 h</b> )	10.7 ( <b>40 h</b> )	0 (24.1 h)	> 100 (24.7 h)
$K_{AP}$ (nM)	0 (17.6 h)	0.99 (19.9 h)	0.89 (29 h)	> 100 (20.5 h)	0 (22.8 h)	1.15 (17.5 h)	0 (22.4 h)	> 100 (24.7 h)
$K_{AC}$ (nM)	0 (22.3 h)	1.06 (14.3 h)	0 (24.2 h)	> 100 (20.5 h)	0 (18 h)	1.24 (21.3 h)	0 (23.5 h)	> 100 (24.7 h)
$K_{IB}$ (nM)	1.46 ( <b>20.3 h</b> )	6.4 ( <b>44.9 h</b> )	0.45 ( <b>12.5 h</b> )	1.12 ( <b>29 h</b> )	1.7 (21 h)	> 100 (25.4 h)	0.15 ( <b>7.6 h</b> )	12.5 ( <b>42.5 h</b> )
$k_{dmB}$ (h <sup>-1</sup> )	0 (26.6 h)	0.023 (20.7 h)	0 ( <b>32 h</b> )	0.09 ( <b>13.7 h</b> )	0.003 (31.2 h)	0.015 (21.9 h)	0 ( <b>26.7 h</b> )	0.24 ( <b>11.1 h</b> )
$k_{dmc}$ (h <sup>-1</sup> )	0 (23.8 h)	0.22 (16.7 h)	0 (25.5 h)	> 100 (20.5 h)	0 (24.6 h)	0.05 (21.2 h)	0 (24 h)	> 100 (24.7 h)
$k_{dmp}$ (h <sup>-1</sup> )	0 (24.4 h)	0.23 (18.8 h)	0 (28.7 h)	> 100 (20.5 h)	0 (23.5 h)	0.06 (17.8 h)	0 (24 h)	> 100 (24.7 h)
$k_{dn}$ (h <sup>-1</sup> )	0 (24.8 h)	0.18 (17.7 h)	0 ( <b>30 h</b> )	0.11 ( <b>15.2 h</b> )	0 (24.7 h)	0.05 (21.6 h)	0 (25 h)	0.17 (14.1 h)
$k_{dnc}$ (h <sup>-1</sup> )	0 (22.3 h)	> 100 (25 h)	0 (25.5 h)	> 100 (20.5 h)	0 (23.8 h)	11.7 (24.4 h)	0 (24 h)	> 100 (24.7 h)
$K_d$ (nM)	0.001 (22.3 h)	> 100 (30.8 h)	0 (17.9 h)	14.5 (26.3 h)	0.00006 (21.8 h)	12.7 (27.5 h)	0 (26.4 h)	> 100 (23 h)
$K_{dp}$ (nM)	0 (23.5 h)	> 100 (22.5 h)	0 (26.3 h)	> 100 (22.7 h)	0 (25.6 h)	> 100 (22 h)	0 (23.3 h)	> 100 (24.5 h)
$K_p$ (nM)	0.0007 (25.9 h)	> 100 (32.8 h)	0 (31.9 h)	2.1 (22.3 h)	0.003 ( <b>47.9 h</b> )	7.4 ( <b>26.2 h</b> )	0.002 ( <b>29.4 h</b> )	0.56 ( <b>13.9 h</b> )
$K_{mB}$ (nM)	0 ( <b>22.7 h</b> )	4 ( <b>43.3 h</b> )	0 (21 h)	4.1 (31 h)	0.7 (22.5 h)	3.1 (33 h)	0 (22.4 h)	11.8 (25.3 h)
$K_{mC}$ (nM)	0.0004 (27.6 h)	> 100 (22.3 h)	0 (24 h)	> 100 (24 h)	0 (28 h)	> 100 (18 h)	0 (24.9 h)	> 100 (23.1 h)
$K_{mP}$ (nM)	0.002 (24.6 h)	> 100 (17.6 h)	0.002 (19 h)	2.1 (30 h)	0.002 (20 h)	> 100 (23 h)	0 (24.9 h)	> 100 (20.8 h)
$k_{sB}$ (h <sup>-1</sup> )	<b>0.06</b> (20.7 h)	<b>0.66</b> (23.4 h)	<b>0.17</b> (20.8 h)	<b>0.2</b> (24.3 h)	<b>0.16</b> (22 h)	<b>0.35</b> (32.5 h)	<b>0.16</b> (24.8 h)	> 100 (14.8 h)
$k_{sC}$ (h <sup>-1</sup> )	0.15 (33.2 h)	> 100 (22.2 h)	0 (20.5 h)	> 100 (24 h)	0.39 (28 h)	> 100 (18 h)	0 (24.7 h)	> 100 (23.1 h)
$k_{sP}$ (h <sup>-1</sup> )	0.06 ( <b>35 h</b> )	> 100 ( <b>17.6 h</b> )	0 (20.5 h)	1.25 (27 h)	0.7 (24.7 h)	> 100 (22.9 h)	0 (24.7 h)	> 100 (20.8 h)
$m$	1.4 (21.8 h)	> 100 (25.4 h)	1.7 (27.2 h)	> 100 (24.7 h)	2.5 (21.3 h)	> 100 (25.4 h)	1.1 (30.9 h)	> 100 (23.8 h)
$n$	1.07 (25.7 h)	> 100 (23.5 h)	0 (17.2 h)	> 100 (24.6 h)	2.3 (26 h)	> 100 (22.9 h)	0 (24.7 h)	> 100 (24.1 h)
$V_{1B}$ (nMh <sup>-1</sup> )	0 (25.2 h)	1.3 (20.5 h)	0 ( <b>26.7 h</b> )	<b>6.9</b> ( <b>13.6 h</b> )	0 (27.3 h)	0.98 (21.6 h)	0 ( <b>24.7 h</b> )	25.1 ( <b>11.8 h</b> )
$V_{1C}$ (nMh <sup>-1</sup> )	0 (23.5 h)	> 100 (30.6 h)	0 (24.1 h)	> 100 (17.7 h)	0 (22.7 h)	9.3 (24.3 h)	0 (23.5 h)	> 100 (24.7 h)
$V_{1P}$ (nMh <sup>-1</sup> )	0 (23.2 h)	> 100 (28.9 h)	0 (24.2 h)	> 100 (18.8 h)	0 (23.1 h)	8.5 (19.2 h)	0 (23.1 h)	> 100 (24.7 h)

$V_{1PC}$ (nMh $^{-1}$ )	0 (24.5 h)	22 (23.5 h)	0 (26.2 h)	> 100 (20.2 h)	0 (23.4 h)	11.6 (18.8 h)	0 (22.7 h)	> 100 (24.7 h)
$V_{2B}$ (nMh $^{-1}$ )	0 (23.4 h)	> 100 (25.2 h)	0 (23.6 h)	> 100 (26.6 h)	0 (23.1 h)	> 100 (27.3 h)	0 (23.9 h)	> 100 (24.7 h)
$V_{2C}$ (nMh $^{-1}$ )	0 (23.8 h)	> 100 (23.5 h)	0 (24.2 h)	> 100 (24.1 h)	0 (23.7 h)	> 100 (22.8 h)	0 (24.2 h)	> 100 (23.6 h)
$V_{2P}$ (nMh $^{-1}$ )	0 (23.8 h)	> 100 (23.3 h)	0 (24 h)	> 100 (24.2 h)	0 (24.3 h)	> 100 (23.1 h)	0 (24.3 h)	> 100 (23.2 h)
$V_{2PC}$ (nMh $^{-1}$ )	0 (23.7 h)	> 100 (24.5 h)	0 (23.5 h)	> 100 (26.1 h)	0 (24.2 h)	> 100 (23.4 h)	0 (24.2 h)	> 100 (22.7 h)
$V_{3B}$ (nMh $^{-1}$ )	0 (25.76 h)	1.3 (18 h)	0 ( <b>30.1 h</b> )	3.3 ( <b>14.6 h</b> )	0 (30.3 h)	1.2 (22.5 h)	0 (18.7 h)	5.6 (12.9 h)
$V_{3PC}$ (nMh $^{-1}$ )	0 (24.9 h)	16 (22.6 h)	0 (26.3 h)	> 100 (20.2 h)	0 (25.4 h)	3.9 (20.2 h)	0 (23.7 h)	> 100 (24.7 h)
$V_{4B}$ (nMh $^{-1}$ )	0 (23.1 h)	> 100 (24.7 h)	0 (23.4 h)	> 100 (29.9 h)	0.06 (21.8 h)	> 100 (30.3 h)	0 (24.4 h)	> 100 (18.8 h)
$V_{4PC}$ (nMh $^{-1}$ )	0 (23.6 h)	> 100 (24.9 h)	0 (23.6 h)	> 100 (26.3 h)	0 (24.4 h)	> 100 (25.4 h)	0 (24.2 h)	> 100 (23.7 h)
$V_{phos}$ (nMh $^{-1}$ )	0 (22.4 h)	3.2 (26.5 h)	0.14 (26.7 h)	> 100 (20.3 h)	0.33 (22.9 h)	3.3 (20.8 h)	0 (19.8 h)	> 100 (24.7 h)
$v_{dBC}$ (nMh $^{-1}$ )	0 (23.9 h)	> 100 (23.7 h)	0 (25.6 h)	> 100 (24.9 h)	0 (25.5 h)	> 100 (23.1 h)	0 (24 h)	> 100 (24 h)
$v_{dBN}$ (nMh $^{-1}$ )	0 (23.6 h)	> 100 (23.7 h)	0 (25.4 h)	> 100 (24.7 h)	0 (23.9 h)	4.7 (21.7 h)	0 (24.2 h)	> 100 (24.3 h)
$v_{dCC}$ (nMh $^{-1}$ )	0 (23.8 h)	> 100 (23.8 h)	0 (25.4 h)	> 100 (25.3 h)	0 (23.8 h)	> 100 (23.7 h)	0 (24.1 h)	> 100 (24.2 h)
$v_{dIN}$ (nMh $^{-1}$ )	0 (34 h)	> 100 (23.4 h)	0.55 (27 h)	> 100 (23.3 h)	0.38 (27.2 h)	> 100 (22.4 h)	0 (24 h)	> 100 (24.2 h)
$v_{dPC}$ (nMh $^{-1}$ )	0 (23.6 h)	> 100 (23.8 h)	0 (25.5 h)	> 100 (25.3 h)	0 (23.7 h)	> 100 (24.3)	0 (23.9 h)	> 100 (24.2 h)
$v_{dpCC}$ (nMh $^{-1}$ )	0 (23.9 h)	> 100 (23.8 h)	0 (25.4 h)	> 100 (24 h)	0 (23.7 h)	> 100 (24.2 h)	0 (24 h)	> 100 (24.2 h)
$v_{dpCN}$ (nMh $^{-1}$ )	0 (23.6 h)	> 100 (23.7 h)	0 (25.7 h)	> 100 (23.6 h)	0 (23.8 h)	> 100 (24.3 h)	0 (23.8 h)	> 100 (24.2 h)
$v_{mB}$ (nMh $^{-1}$ )	<b>0.65</b> (27.1 h)	<b>0.89</b> (21 h)	<b>0.35</b> (34 h)	<b>1.0</b> ( <b>14 h</b> )	<b>0.42</b> (33 h)	<b>0.53</b> (22 h)	<b>0.03</b> ( <b>58.7 h</b> )	<b>0.76</b> ( <b>13.1 h</b> )
$v_{mC}$ (nMh $^{-1}$ )	0 (22.2 h)	1.4 (16.7 h)	0 (24.5 h)	> 100 (20.5 h)	0 (18 h)	1.06 (21 h)	0 (23.1 h)	> 100 (24.7 h)
$v_{mP}$ (nMh $^{-1}$ )	0 (17.6 h)	1.5 (19.8 h)	0.85 (32.5 h)	> 100 (20.5 h)	0 (23 h)	0.83 (17.5 h)	0 (20.8 h)	> 100 (24.7 h)
$v_{sB}$ (nMh $^{-1}$ )	<b>0.9</b> (20.5 h)	<b>1.19</b> (26.9 h)	<b>0.78</b> ( <b>15.5 h</b> )	<b>1.6</b> ( <b>33 h</b> )	<b>0.48</b> (22.1 h)	<b>0.57</b> (32.2 h)	0.22 ( <b>17.6 h</b> )	> 100 ( <b>82.5 h</b> )
$v_{sC}$ (nMh $^{-1}$ )	0.76 (18.4 h)	> 100 (22.2 h)	0 (20.5 h)	> 100 (24.5 h)	1.18 (22.8 h)	> 100 (18 h)	0 (24.7 h)	> 100 (23.2 h)
$v_{sP}$ (nMh $^{-1}$ )	1.06 (21.6 h)	> 100 (17.6 h)	0 (20.5 h)	1.55 (33.2 h)	0.92 (18.5 h)	> 100 (22.8 h)	0 (24.7 h)	> 100 (20.7 h)
$v_{stot}$ (nMh $^{-1}$ )	<b>0.92</b> ( <b>21.2 h</b> )	<b>1.14</b> ( <b>45.2 h</b> )	<b>0.7</b> ( <b>16.6 h</b> )	<b>1.11</b> ( <b>33.2 h</b> )	<b>0.95</b> (19 h)	<b>1.14</b> (32.5 h)	<b>0.34</b> (17.7 h)	<b>1.84</b> (31.2 h)
$k_{stot}$ (h $^{-1}$ )	<b>0.55</b> (25.9 h)	<b>1.96</b> (32.6 h)	<b>0.51</b> (19 h)	<b>1.6</b> (28.4 h)	<b>0.81</b> (22.6 h)	<b>1.72</b> (28.2 h)	<b>0.16</b> ( <b>24.9 h</b> )	<b>5.5</b> ( <b>10.2 h</b> )

Four distinct sets of parameter values yielding an autonomous period in constant darkness close to 24 h are considered (see Table 1). Parameters were varied one at a time, while holding other parameters at their basal values listed in Table 1. For each parameter we give the lower and upper bounds of the domain of sustained oscillations, as well as the period (in parentheses) in these points. The variation of the period over the whole oscillatory range is shown in Figs. 4–8 by bifurcation diagrams for a selected choice of parameters. To emphasize the parameters to which the system is most sensitive, the parameter and the period are indicated in bold type when the period at the two boundaries of the oscillatory domain varies by a factor larger than 2. To indicate a second type of sensitivity, the parameter and its lower and upper values at the boundaries of the oscillatory domain are indicated in bold type when the domain of oscillations is particularly narrow. The data were obtained by numerical integration of Eqs. (1)–(16).

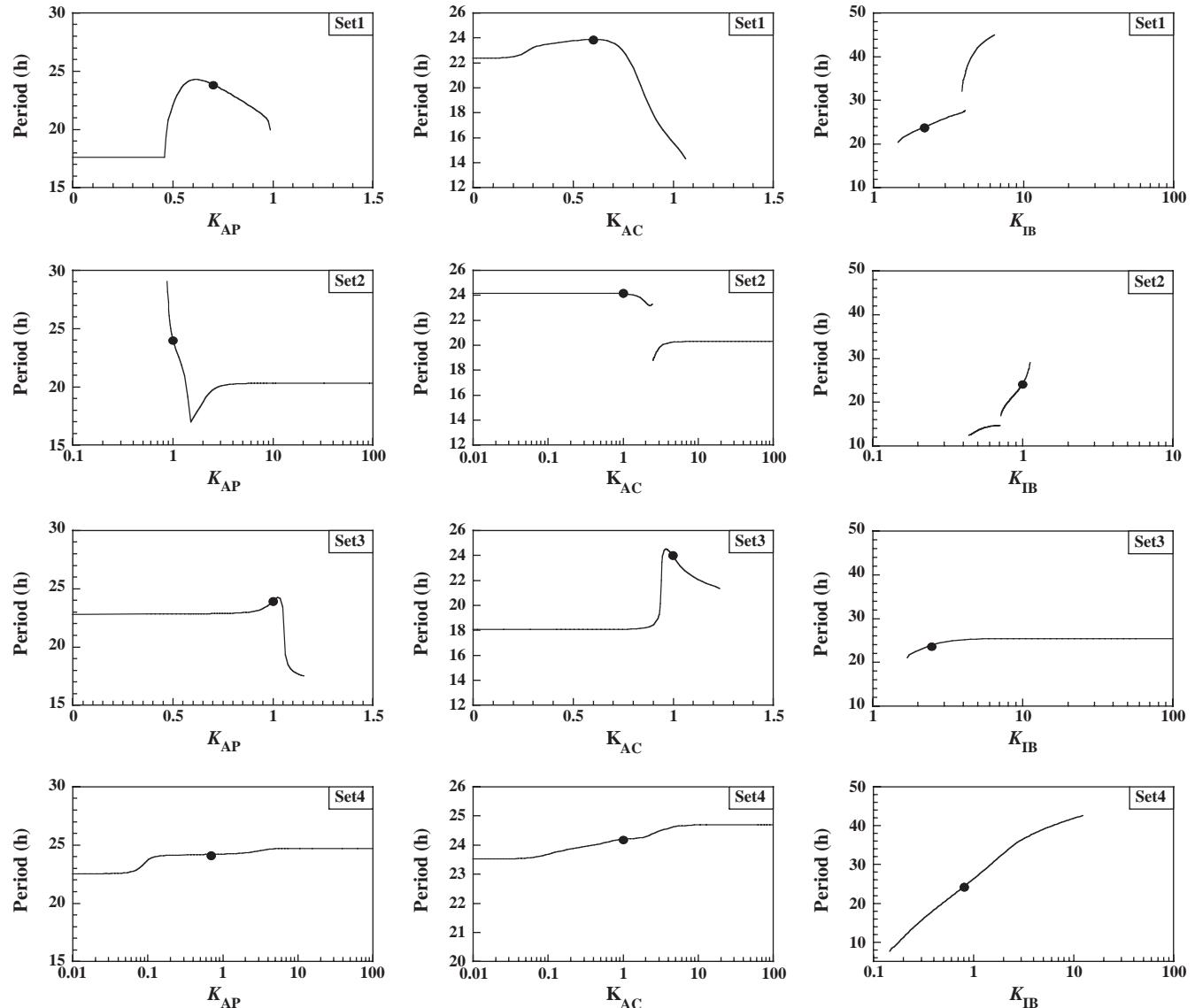


Fig. 4. Bifurcation diagrams showing the variation of the period as a function of the activation constants ( $K_{AP}$ ,  $K_{AC}$ ) and inhibition constant ( $K_{IB}$ ). The curves were obtained for parameter sets 1–4 (top to bottom rows) listed in Table 1, by numerical integration of Eqs. (1)–(16). In all panels in Figs. 4–8, the dot on each curve relates to the basal parameter value listed in Table 1. Two stable rhythms sometimes coexist over a small range of the parameter values (see, for example, the bifurcation diagram established as a function of  $K_{IB}$  for parameter sets 1 or 2). This phenomenon, referred to as birhythmicity, has also been observed in a 10-variable model for circadian rhythms in *Drosophila* (Leloup and Goldbeter, 1998).

in contrast, may pass through a maximum or/and a minimum. The diagrams show that the variation of the period over the oscillatory range of a given parameter and the very shape of this curve may markedly depend on the other parameter values. This is particularly striking for parameter  $K_{AP}$  (Fig. 4, first column): for parameters sets 1 and 3, as  $K_{AP}$  increases, the period at first does not vary much, then increases, passes through a maximum and eventually decreases until oscillations disappear above a critical value of  $K_{AP}$ . In contrast, for parameter set 2, the reverse situation is observed. Yet another shape for period vs  $K_{AP}$  relationship is observed for parameter set 4; the range of  $K_{AP}$  values producing

oscillations is also larger in that case. This difference between the various parameter sets 1–4 can be related to the particular mechanism underlying the oscillations. The dependence of the period on parameters  $k_{SP}$ ,  $k_{SC}$  and  $k_{SB}$  (Fig. 7) also differs according to the parameter set considered. For these parameters, and for other parameters such as  $V_{phos}$  (Fig. 8) and  $K_{AP}$  (Fig. 4) the domain of values producing oscillations is larger for parameter set 4 as compared to sets 1–3. In the case of parameter  $V_{phos}$  (Fig. 8) the oscillatory domain is bounded by a higher critical value for parameter set 1, by a lower critical value for parameter set 2, by both a lower and a higher critical value for parameter set 3,

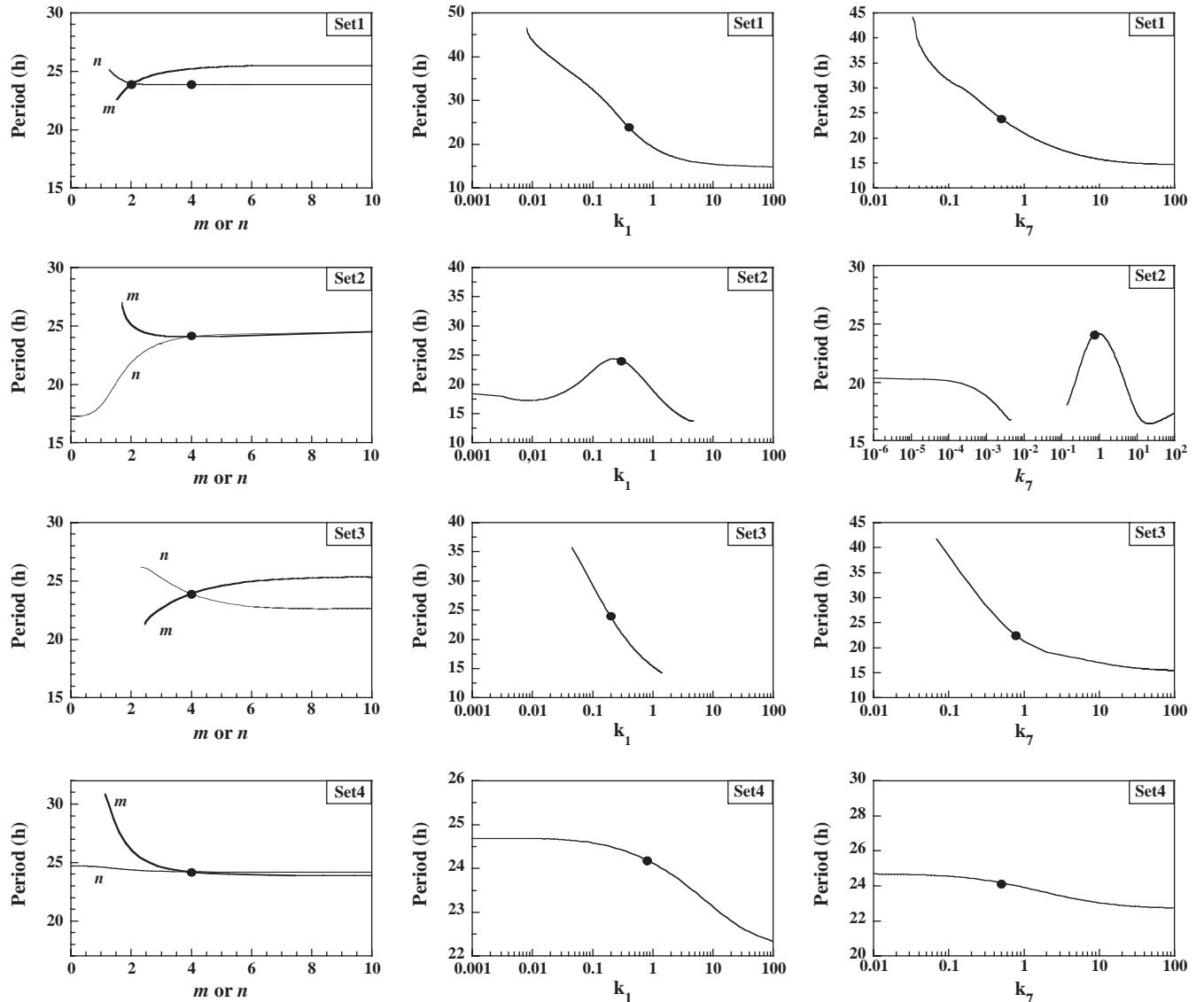


Fig. 5. Bifurcation diagrams showing the variation of the period as a function of the rate constants  $k_1$  and  $k_7$ , and of the degrees of cooperativity  $m$  and  $n$ . The curves were obtained for parameter sets 1–4, as indicated in Fig. 4.

while no critical value bounding the oscillatory domain is found for parameter set 4.

Of particular interest is the situation sometimes encountered for parameter  $k_7$  (Fig. 5, right column), which measures the rate of formation of the inactive complex between CLOCK–BMAL1 and PER–CRY. Thus, for parameter set 2—but not for the other parameter sets considered in Table 1—the bifurcation diagram as a function of  $k_7$  can present two distinct ranges of values producing sustained oscillations, separated by an intermediary range of values corresponding to evolution toward a stable steady state (see Fig. 9A, which reproduces the panel in the second row of the middle column in Fig. 5). As further explained in the Discussion, the existence of two interconnected branches of oscillations illustrates the existence of two

distinct oscillatory mechanisms that may sometimes operate independently from each other. The right branch of the bifurcation diagram in Fig. 9A is associated with the indirect negative feedback exerted by PER–CRY, as indicated by the disappearance of this branch in Fig. 9B when the expression of the *Per* and *Cry* genes becomes constitutive ( $K_{AP} = K_{AC} = 0$ ). The left branch of the bifurcation diagram in Fig. 9A is associated with the oscillatory mechanism based on BMAL1 negative autoregulatory feedback, as shown by its disappearance in Fig. 9C when  $m = 2$ : then the autoregulatory negative feedback of BMAL1, for the parameter set considered, ceases to produce autonomous oscillations. Both in Figs. 9B and C the other oscillatory mechanism remains active when either the first or the second oscillatory mechanism is silenced.

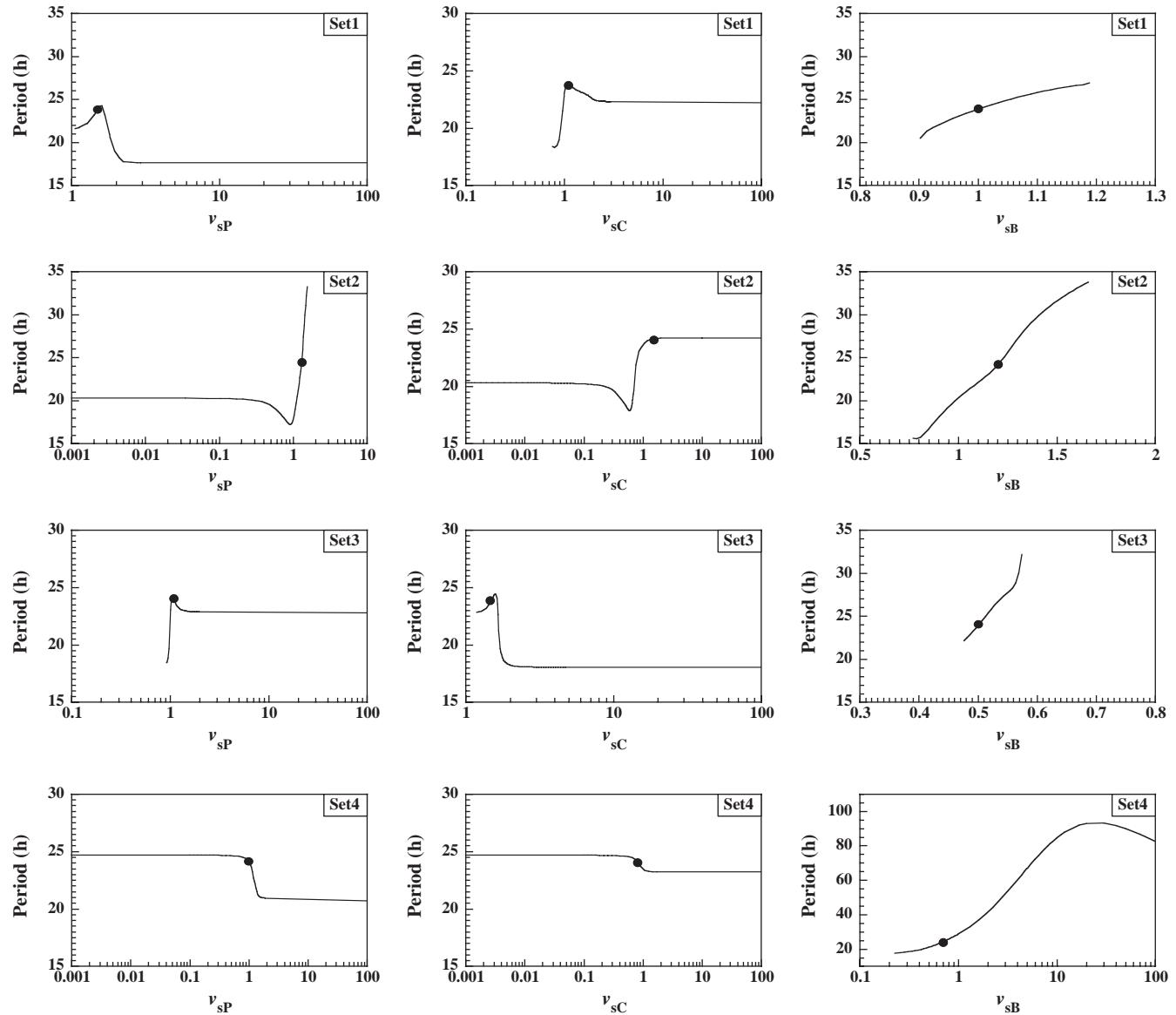


Fig. 6. Bifurcation diagrams showing the variation of the period as a function of the maximum rates  $v_{sp}$ ,  $v_{sc}$  and  $v_{sb}$  measuring the expression of the *Per*, *Cry* and *Bmal1* genes, respectively. The curves were obtained for parameter sets 1–4, as indicated in Fig. 4.

## 9. Phase upon entrainment of circadian oscillations by light-dark cycles

To probe for entrainment of the circadian clock by LD cycles, we must incorporate the effect of light on *Per* expression (Zylka et al., 1998). In continuous darkness the maximum rate of *Per* expression,  $v_{sp}$ , remains at a low, constant value. In LD, this rate varies periodically, e.g. as a square wave, going from a constant low value during the dark phase up to a higher constant value  $v_{spmax}$  during the light phase. In such conditions, entrainment by a 12:12 LD cycle (12 h of light followed by 12 h of darkness) can be obtained over a range of  $v_{spmax}$  values (Leloup and Goldbeter, 2003).

We previously showed that the phase of the oscillations after entrainment is sensitive to the choice of parameter values, and that different phases can even be obtained in LD for parameter values yielding comparable periods of circadian oscillations in DD (Leloup and Goldbeter, 2003). The phase is particularly sensitive to changes in parameters that control the level of CRY protein and *Cry* mRNA. This was previously shown for the case of parameter  $K_{AC}$ , the equilibrium constant describing the activating effect of CLOCK–BMAL1 on *Cry* expression, and is now illustrated in Fig. 10 for the case of parameter  $v_{mc}$ , which measures the maximum rate of degradation of *Cry* mRNA. An example of this situation is illustrated in Fig. 10B, where the only

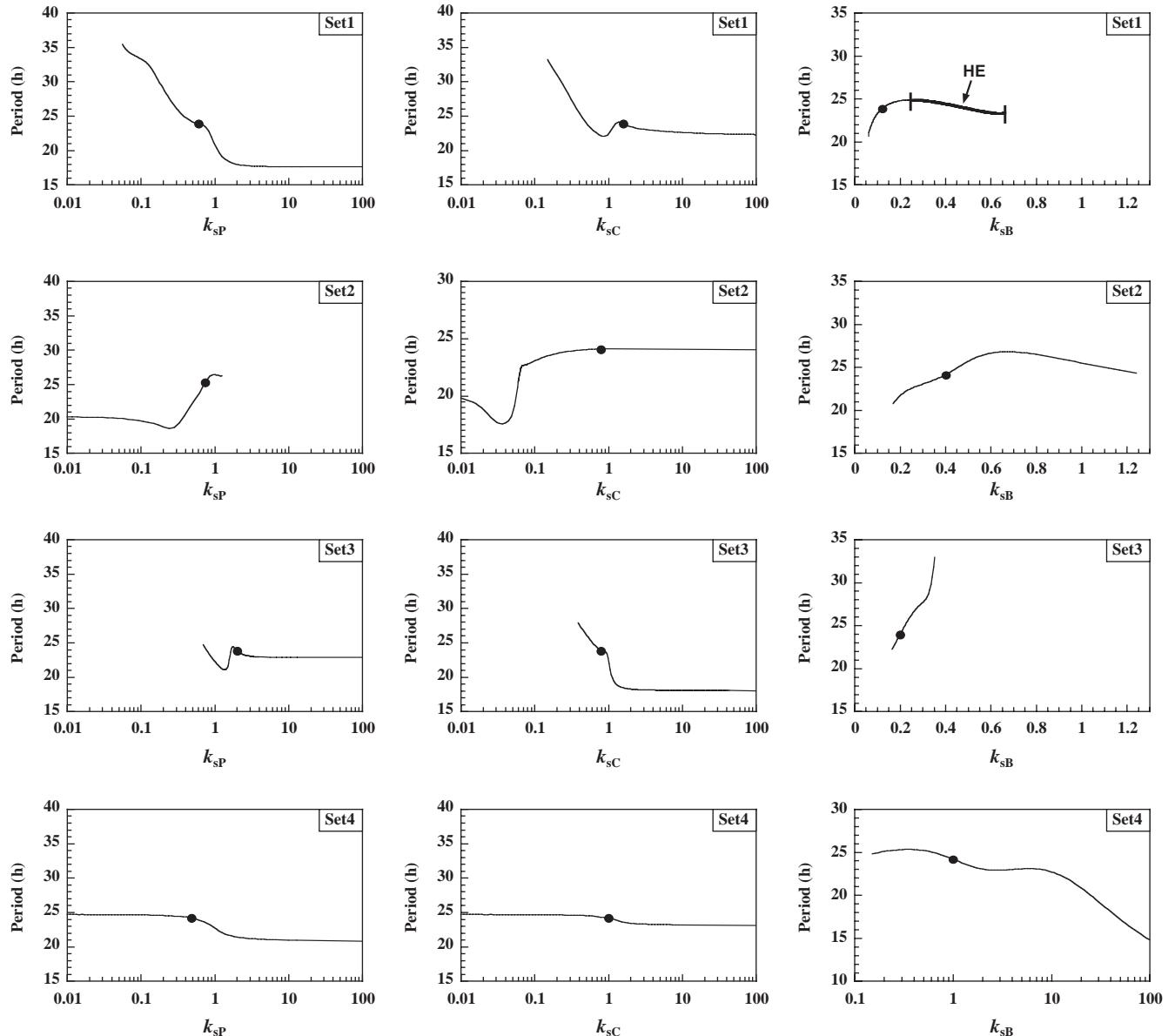


Fig. 7. Bifurcation diagrams showing the variation of the period as a function of the constants  $k_{sp}$ ,  $k_{sc}$  and  $k_{sb}$  measuring, respectively, the rate of synthesis of the PER, CRY and BMAL1 proteins. The curves were obtained for parameter sets 1–4, as indicated in Fig. 4. A coexistence between a stable steady state and a stable periodic regime (which phenomenon is known as hard excitation) can sometimes be observed over a restricted range of parameter values, e.g. for parameter set 1 as a function of parameter  $k_{sb}$ . The region of hard excitation corresponds to the part of the curve shown as a thick solid line and marked HE. Domains of hard excitation in the other bifurcation diagrams in Figs. 4–8 may well exist, but they were not characterized.

difference with respect to Fig. 10A is a 10% change in parameter  $v_{mC}$ . The autonomous period in DD is 23.85 and 23.70 h in Figs. 10A and B, respectively. The phase of *Per* mRNA is delayed by about 10 h in the latter case, so that *Per* mRNA reaches its maximum during the D phase instead of peaking in the L phase.

## 10. Discussion

The computational model for the mammalian circadian clock presented in a recent publication (Leloup and

Goldbeter, 2003), and considered here in further detail, incorporates the main clock components identified so far, namely, the *Per*, *Cry*, *Clock* and *Bmal1* gene products. The model accounts for the occurrence of autonomous circadian oscillations, in conditions corresponding to continuous darkness, with antiphase relationships between *Per* and *Cry* mRNAs on the one hand, and *Bmal1* mRNA on the other hand. Entrainment by LD cycles can also occur, via the induction of *Per* expression in the light phase. Similar results are obtained (Leloup and Goldbeter, 2003) when incorporating into the model the REV-ERB $\alpha$  protein, which

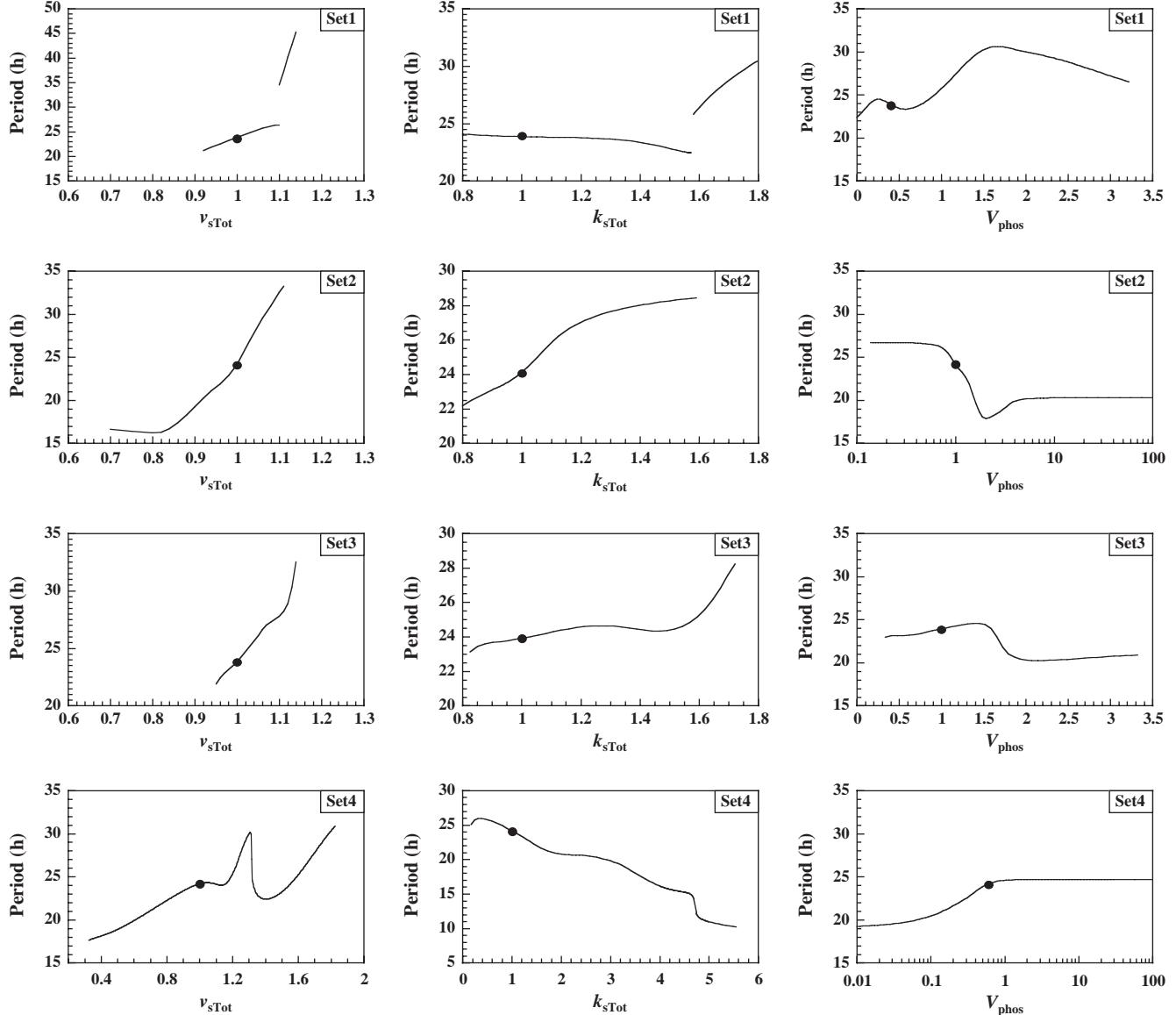


Fig. 8. Bifurcation diagrams showing the variation of the period as a function of the global parameters  $v_{sTot}$ ,  $k_{sTot}$  and  $V_{phos}$  related, respectively, to the rates of gene expression and protein synthesis for PER, CRY and BMAL1, and to the rate of phosphorylation of all forms of the PER protein. The curves were obtained for parameter sets 1–4, as indicated in Fig. 4. (See Table 1 for the definition of  $v_{sP}$ ,  $v_{sC}$ ,  $v_{sB}$  as a function of  $v_{sTot}$ ;  $k_{sP}$ ,  $k_{sC}$ ,  $k_{sB}$  as a function of  $k_{sTot}$ ; and  $V_{1P}$ ,  $V_{1PC}$ ,  $V_{3PC}$  as a function of  $V_{phos}$ .)

mediates the negative feedback of BMAL1 on the expression of the *Bmall* gene (Preitner et al., 2002). Although it represents a step toward a detailed computational model for the mammalian clock, some gene products, which have been shown to play a role, are not included, e.g. the DEC1 and DEC2 proteins (Honma et al., 2002; Kawamoto et al., 2004), and the TIM protein, which also appears to have a role in mammals (Barnes et al., 2003).

A conspicuous feature of the model for the mammalian clock is that it reveals the possible existence of multiple sources of oscillatory behavior. Thus, even in the absence of *Per* mRNA or PER protein, the model

predicts the possibility of sustained oscillations (Leloup and Goldbeter, 2003; see also Fig. 3B), which are due to the negative autoregulatory feedback exerted by CLOCK–BMAL1 on the expression of the *Bmall* gene. However, the period of these oscillations may not necessarily be circadian, although we may expect that it is measured in hours because the rhythm relies on the regulation of gene expression. It appears, therefore, that two oscillators are coupled within the circadian control system. The first one relies on the indirect negative feedback exerted through the inhibitory binding of PER–CRY to the CLOCK–BMAL1 complex which activates the expression of *Per* and *Cry*. The second

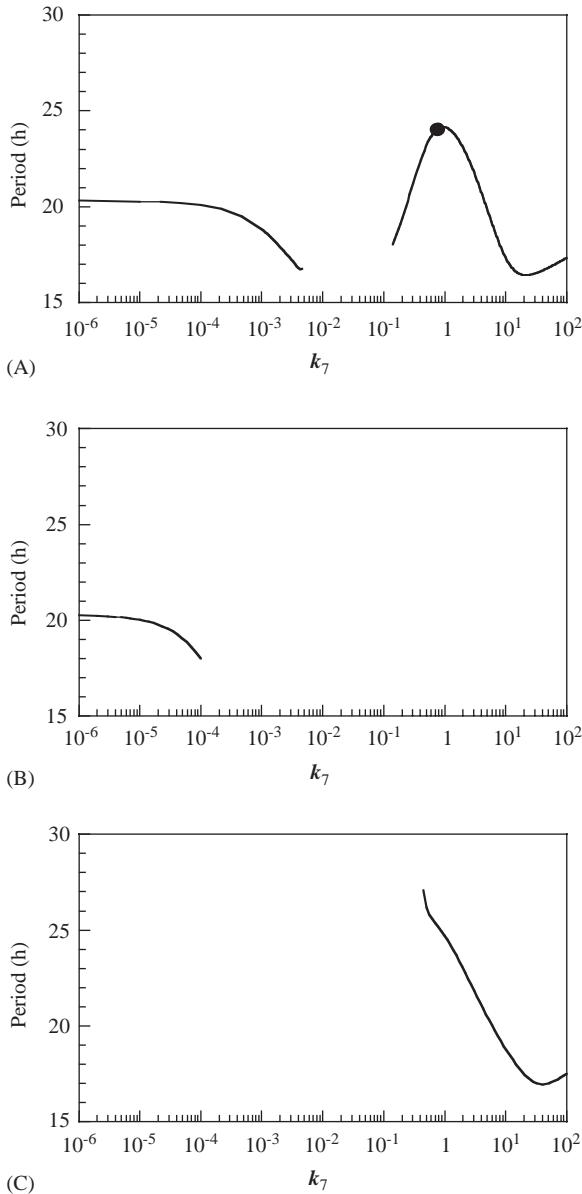


Fig. 9. Additional evidence for multiple oscillatory mechanisms. The bifurcation diagrams show the variation of the period as a function of the rate constant  $k_7$  for the formation of the inactive complex between PER–CRY and CLOCK–BMAL1. Panel A shows the occurrence of distinct regions of oscillations as a function of parameter  $k_7$  for parameter set 2. In panel B, only the left branch of the oscillatory domain remains when the constants  $K_{AP}$  and  $K_{AC}$  characterizing the activation by BMAL1 of *Per* and *Cry* expression are put equal to zero (the mechanism of oscillations based on the negative feedback loop involving PER–CRY and CLOCK–BMAL1 is then silenced). In panel C, only the right branch of the oscillatory domain is retained when the degree of cooperativity  $m$  is equal to two (the second mechanism of oscillations, based on the negative feedback loop involving CLOCK–BMAL1, is then inoperative). The curves were obtained by numerical integration of Eqs. (1)–(16). In panel A, the dot relates to the basal parameter value listed in Table 1.

mechanism capable of generating sustained oscillations is based on the negative feedback exerted by CLOCK–BMAL1, via REV-ERB $\alpha$ , on the expression of the *Bmal1* gene.

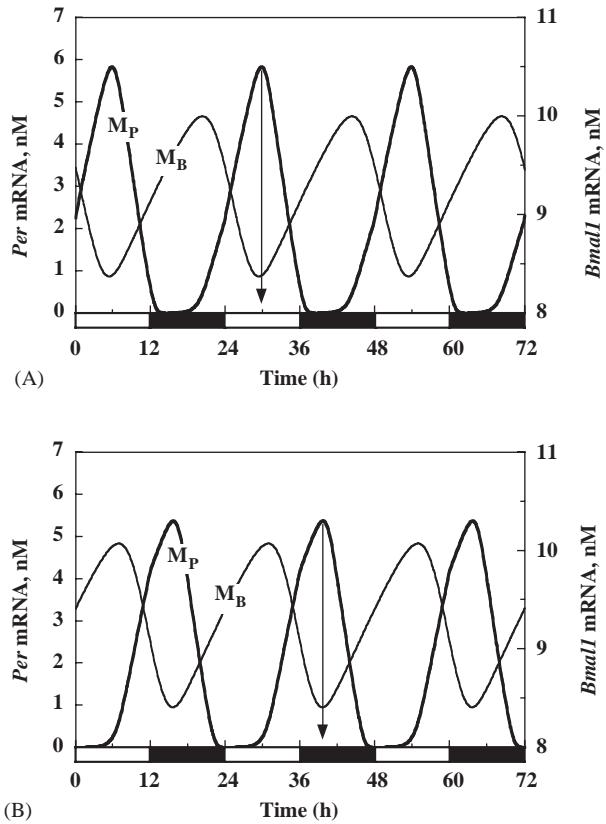


Fig. 10. Oscillations of the mRNAs after entrainment by 24-h light–dark (LD) cycles. (A) The peak in *Per* mRNA ( $M_P$ ) occurs in the middle of the light phase. (B) Oscillations are delayed by about 10 h and the peak in *Per* mRNA occurs in the dark phase when the value of parameter  $v_{mC}$  is decreased from 1.0 to 0.9 nM/h. Other parameter values correspond to the parameter set 1 listed in Table 1. In both cases the peak in  $M_P$  remains in antiphase with the peak in *Bmal1* mRNA ( $M_B$ ). The maximum value of the rate of *Per* expression,  $v_{SP}$ , varies in a square-wave manner such that it remains at a constant low value of 1.5 nM/h during the 12h-long dark phase (black rectangle), and is raised up to the high value of 1.8 nM/h during the 12h-long light phase (white rectangle). The curves have been obtained by numerical integration of Eqs. (1)–(16). The autonomous period is 23.8 h in A and 23.7 h in B.

The possible existence of a second oscillatory mechanism does not imply that it is functional. Indeed, parameter values could be such that the second mechanism remains silent while oscillations primarily originate from the first mechanism. As illustrated in Fig. 3, when preventing oscillations involving the first mechanism, by setting  $k_{SP} = 0$  (no PER synthesis), the system may either evolve to a stable steady state (Fig. 3A) or to sustained oscillations (Fig. 3B), depending on parameter values. The latter rhythm can only be due to the second oscillatory mechanism. Experiments showing circadian oscillations in *Rev-Erb $\alpha$*  knockout mice (Preitner et al., 2002) suggest that the autoregulatory feedback by BMAL1 is not required for periodic behavior, and that the first mechanism based on PER suffices to produce circadian oscillations. This result is

accounted for in the model only for some choices of parameter values, e.g. for parameter set 3: oscillations then occur even at very large values of  $K_{IB}$ , such that the negative autoregulatory feedback by BMAL1 does not operate (see Fig. 4 and Table 2).

The unmasking of a second oscillatory mechanism is apparent not only in Fig. 3 but also in some of the data presented in Figs. 4–8 and Table 2. Among the four sets of parameter values considered, which all yield circadian oscillations, parameter sets 2 and 4 can still produce oscillations when the first oscillatory mechanism ceases to be functional. Arresting oscillations due to the first mechanism can be achieved in a number of ways: setting equal to zero the synthesis of either PER ( $k_{sP} = 0$ ; see Figs. 3 and 7) or CRY ( $k_{sC} = 0$ ; see Fig. 7), or the synthesis of their mRNAs ( $v_{sP} = 0$  or  $v_{sC} = 0$ ; see Fig. 6), or setting to very high values (larger than 100) the degradation rate of these mRNAs,  $v_{mP}$  or  $v_{mC}$ , or the constants  $K_{AP}$  and  $K_{AC}$  (Fig. 4) characterizing the activation by BMAL1 of *Per* and *Cry* expression. Unmasking the second oscillator, based on negative autoregulation by BMAL1, can further be achieved by setting to zero the rate of entry of PER–CRY into the nucleus,  $k_1$  (see Fig. 5), the rate of formation of the complex between PER and CRY,  $k_3$ , the rate of formation of the inactive complex between PER–CRY and CLOCK–BMAL1  $k_7$  (see Figs. 5 and 9A). When one of the oscillators is silenced, the period approaches that of the active oscillator. This can be seen in Table 2 for parameter sets 2 and 4, where the period of the BMAL1 oscillator approaches 20.5 h for parameter set 2 and 24.7 h for parameter set 4. Likewise, for parameter set 3, the oscillations due to the PER–CRY negative loop continue when the BMAL1 oscillator is silenced, with a period approaching 25.4 h as  $K_{IB}$  increases (see Fig. 4 and Table 2).

The methods we used for silencing the first or second oscillator are not equivalent. Thus, there are many ways to quench the PER oscillator: preventing expression of the *Per* and *Cry* genes, making this expression constitutive, preventing entry of PER and CRY into the nucleus or the formation of the PER–CRY complex or of the complex between PER–CRY and CLOCK–BMAL1. In contrast, the BMAL1 oscillator can be silenced in only one way, by making the expression of *Bmall* constitutive.

It is often not possible to totally uncouple the two oscillators. Thus, in Fig. 9, when one of the two branches present in Panel A is suppressed in Panels B and C, the remaining branch has a different appearance than in Panel A. For example, in Fig. 9B, suppression of the PER oscillatory mechanism leads to a shrinking of the range of values of  $k_7$  producing oscillations due to the BMAL1 negative feedback loop. When the *Per* and *Cry* expression becomes constitutive (Fig. 9B), the PER and CRY proteins pump BMAL1 to form the complex,

and therefore alter the amount of BMAL1 available for negative autoregulation. Thereby PER and CRY continue to influence the BMAL1 oscillator at values of  $k_7$  above  $10^{-4}$  in Fig. 9B: because the amount of PER and CRY is larger, the range of values of  $k_7$  producing oscillations is smaller than in Fig. 9A.

Even if the oscillator based on BMAL1 negative autoregulation does not appear to be functional in physiological conditions, as seen by the arrhythmic behavior of *Cry* or *Per* knockout mice (van der Horst et al., 1999; Zheng et al., 2001), the possibility of a second oscillatory mechanism in mammals nevertheless holds with the observation that in *mPer1/mPer2*-deficient mice, rhythmicity can sometimes be restored for several days by an extended light pulse (K. Bae and D. Weaver, pers. comm.).

The model schematized in Fig. 1 applies, with few modifications, to circadian rhythms in non-mammalian organisms such as *Drosophila*. Differences pertain to the effect of light, which is to trigger degradation of the TIM protein rather than *Per* expression, and to the partners of PER and CLOCK, which, instead of CRY and BMAL1, are TIM and CYC, respectively, while the role of REV-ERB $\alpha$  is played by VRILLE (Allada et al., 2001; Williams and Sehgal, 2001; Young and Kay, 2001). The finding of multiple oscillatory mechanisms in the mammalian model could thus be linked to experimental evidence for multiple oscillators in *Drosophila* (Yoshii et al., 2002), and might also bear on related observations of multiple rhythms in *Neurospora* (Merritt et al., 1999) and plants (Green and Tobin, 1999).

In our comparative study of the sensitivity of circadian oscillations with respect to the various model parameters we generally found the highest sensitivity for parameters controlling *Bmall* mRNA or BMAL1. The question arises as to how to reconcile this result with the observation that halving BMAL1 level does not impact much on the circadian oscillations (Bunger et al., 2000; Von Gall et al., 2003)? Perhaps the values of  $K_{AP}$  and  $K_{AC}$  are already well below the BMAL1 level, so that in both cases the effect on activation of *mPer* and *mCry* expression is maximal, even at levels of BMAL1 reduced by 50%. We also note that the range of values of BMAL1-related parameters that gives rise to sustained oscillations is larger for parameter set 4 (see Table 2), as also shown by the bifurcation diagram established in Fig. 7 as a function of parameter  $k_{sB}$ . This diagram indicates that in appropriate conditions the model can produce circadian oscillations that remain largely unaffected following a sizeable reduction in the rate of synthesis of the BMAL1 protein.

The peak in *Per* mRNA after entrainment in LD cycles often falls within the L phase (see Fig. 10A). This result, which holds with experimental observations (Reppert and Weaver, 2001), is expected because *Per* expression increases during the light phase. In a

counterintuitive manner, the model indicates that the phase of the oscillations markedly depends on other parameters of the model that are not affected by light. For example, we previously noted that a decrease in the equilibrium constant characterizing *Cry* gene activation by CLOCK–BMAL1 shifts the maximum in *Per* mRNA from the L to the D phase (Leloup and Goldbeter, 2003). Here, we extended this result by showing that a similar effect is obtained with other parameters controlling the level of CRY protein or *Cry* mRNA (e.g. parameter  $v_{MC}$ ; see Fig. 10B). The lability of the phase might explain how even small differences in parameter values could result in different phases of circadian oscillations in LD in the central pacemaker located in SCN cells and in peripheral tissues that are also capable of displaying circadian oscillations (Balsalobre et al., 1998; Schibler et al., 2003; Yoo et al., 2004). The results support the view (Reppert and Weaver, 2002) that the genetic regulatory mechanism of circadian oscillations is similar in both the central and peripheral oscillators, and that the observed differences in phase are of a quantitative rather than qualitative nature.

The level of CRY is also critical for entrainment by LD cycles. We previously showed that when the level of CRY remains too low, free PER builds up during successive light phases, as there is not enough CRY with which to form a complex; consequently, entrainment fails to occur (Leloup and Goldbeter, 2003). This condition was related to the occurrence of the non-24 h sleep–wake syndrome (Richardson and Malin, 1996). We plan to use the model to determine other possible causes that might lead to a loss of entrainment in LD.

In the familial advanced sleep–wake cycle syndrome, the phase of the sleep–wake cycle in LD is advanced by several hours, as a result of a decreased rate of PER phosphorylation (Toh et al., 2001). Such a phase advance can be accounted for by the model for parameter set 1; as in clinical observations (Jones et al., 1999), the advance of the phase in LD then accompanies a decrease in autonomous period as parameter  $V_{phos}$  decreases (Leloup and Goldbeter, 2003). Such a decrease in period can also be observed over parts of the bifurcation diagrams established as a function of  $V_{phos}$  for all parameter sets 1–4 (see Fig. 8), but the figure indicates that the shape of the bifurcation diagrams may vary according to the set of parameter values considered. The model could be used similarly to address the delayed sleep phase syndrome, which is the mirror physiological disorder of the sleep–wake cycle (Ebisawa et al., 2001; Archer et al., 2003). The computational model for circadian oscillations in mammals thus provides us with the unique opportunity to address not only the molecular mechanism of a key biological rhythm but also the dynamical bases of physiological

disorders resulting from perturbations of the human circadian clock.

Understanding the molecular, dynamical bases of circadian rhythm disorders in mammals is of interest besides the link with syndromes pertaining to the sleep–wake cycle. Severe perturbations of circadian rhythms have recently been associated with an increased propensity to develop various forms of cancer (Filipski et al., 2002; Fu et al., 2002). Of particular import is the finding that the circadian clock via CLOCK–BMAL1 directly controls the *wee1* kinase gene involved in cell cycle progression (Matsuo et al., 2003). The effects of the coupling between the circadian clock and the cell cycle biochemical machinery could be explored by means of the present model, when coupling it to a molecular model for the eukaryotic cell cycle (Tyson and Novak, 2001).

One of the most intriguing properties of circadian rhythms, observed in some organisms, is their suppression by a single pulse of light (Engelmann et al., 1978). Early on the phenomenon was related (Kalmus and Wigglesworth, 1960) to the situation of hard excitation (Minorsky, 1962) (or “shock” excitation) in which a stable steady state coexists with stable oscillations. Suppression of circadian rhythms by a light pulse was previously obtained in a model for the *Drosophila* circadian clock, in conditions where a stable steady state coexists with a stable limit cycle (Leloup and Goldbeter, 2001). In such conditions, the system can switch from the oscillatory regime into the basin of attraction of the nonoscillatory regime, as a result of a light-induced, transient increase in the degradation rate of the TIM protein. Similar results are obtained when the light pulse induces a transient increase in the expression of the *Per* gene, as occurs in mammals. The transition to the nonoscillatory state can be reverted by a second light pulse, which restores circadian oscillations.

The coexistence between a stable steady state and a stable limit cycle can also be observed in the model for the mammalian clock governed by Eqs. (1)–(16), as illustrated by the bifurcation diagram established as a function of parameter  $k_{sB}$  for parameter set 1 in Fig. 7 (domains of hard excitation in the other bifurcation diagrams in Figs. 4–8 were not characterized). In these conditions, a 3-h increase by a factor of 4 in *Per* expression triggered by a light pulse can suppress sustained circadian oscillations (Fig. 11). In contrast to the results obtained for circadian rhythm suppression by a light pulse in a 10-variable model for the circadian clock (Leloup and Goldbeter, 2001), starting from the stable steady state, we did not succeed in restoring the rhythm by a second light pulse inducing *Per* expression when varying the duration of the transient increase in  $v_{SP}$  over a 10 h range and when increasing the parameter over a tenfold range with respect to its basal value. This

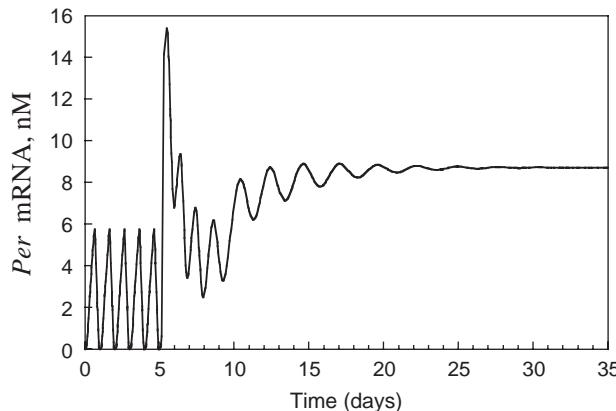


Fig. 11. Suppression of circadian oscillations by a single pulse of light. Parameter  $v_{SP}$  was multiplied by a factor of 4 during 3 h, starting 4 h after the minimum in *Per* mRNA ( $M_P$ ). The curve was established for parameter set 1, using the value  $k_{sB} = 0.5 \text{ h}^{-1}$ . This value lies inside the domain of hard excitation marked HE in the bifurcation diagram shown in Fig. 7 (third column, row 1).

result indicates that in the case considered it is more difficult to restore the rhythm than to suppress it, apparently because the perturbation in only one of the 16 variables does not suffice to bring the system away from the stable steady state to the limit cycle. The generality of this conclusion remains to be tested for other domains of hard excitation that would be found in the parameter space for this model, but we already know from the study of the 10-variable model mentioned above that return from the stable steady state to the limit cycle by perturbation of a single variable is sometimes possible.

The suppression of circadian rhythms by a light pulse, reported for a number of mammalian species (Jewett et al., 1991; Klante and Steinlechner, 1995; Honma and Honma, 1999), could thus operate according to the mechanism illustrated in Fig. 11. In this view, rhythmic behavior should be suppressed in all pacemaker cells. In the alternative view proposed by Winfree (1973, 1980), suppression of overt circadian rhythms originates from the desynchronization induced by the light pulse: all pacemaker cells go back to the unstable, phaseless singularity and may return thereafter to the limit cycle, which they reach with randomly distributed phases. To discriminate between the two possibilities, it would be necessary to determine whether the suppression of circadian rhythms by a light pulse is accompanied by suppression of the rhythm in every pacemaker cell or whether the rhythm eventually resumes with a different phase in each cell. Yet another possible explanation for suppression involves the phase shift of a subpopulation of cells in the suprachiasmatic nuclei (SCN), with respect to another subpopulation of SCN cells that would remain unaffected (Nagano et al., 2003). The phase difference created by the light pulse between

different subpopulations of cells in the SCN could abolish the overt circadian rhythm.

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