

A Model of the Mammalian Circadian Oscillator Including the REV-ERB α Module

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Abstract

Many cellular and physiological processes have been shown to display a rhythm of about 24 hours. This so-called circadian rhythm is based on a system of interlocked negative and positive molecular feedback loops. Here we extend a previous model of the circadian oscillator by including REV-ERB α as an additional component. This new model will allow us to investigate the function of an additional negative feedback loop via REV-ERB α . We obtain circadian oscillations with the correct period and phase relations between clock components. Parameter variations that correspond to clock-gene mutations reproduce experimental results: With parameter variations mimicking the *Bmal1*^{-/-} and the *Per2*^{Brdm1} mutation the oscillations cease to exist. In contrast, the system shows sustained oscillations if we use a parameter set that reflects the *Rev-erba* mutation. The model also accounts for the differential effect of the *Cry1*^{-/-} and *Cry2*^{-/-} mutations on the circadian period. The simulations of the extended model indicate that the original model is robust with respect to the incorporation of the additional component. Depending on the kinetics of the *Per2/Cry* transcriptional activation by BMAL1, an increasing BMAL1 expression leads to either an increase or decrease of the clock period. This indicates that overexpression experiments could help to characterize the impact of BMAL1 on *Per2/Cry* transcription.

Keywords: circadian oscillator, mathematical model, mutants, period, cryptochrome, *rev-erba*, bifurcation analysis

1 Introduction

In almost all organisms, an internal circadian clock with a period of about 24 hours has evolved that allows timekeeping even without external stimuli. This clock organizes the temporal order of molecular, physiological and behavioral processes and thereby contributes to their optimal adaptation to certain daytimes. In several tissues about 10% of mRNA concentrations have been found to be oscillating with a 24 hour period [15], demonstrating the vast influence of the clock on cellular processes. While many tissues contain a molecular clock, in mammals the master clock that coordinates the others is located in the suprachiasmatic nuclei (SCN) in the hypothalamus [14].

The circa 24 hour rhythm is based on cell-autonomous sustained oscillations of mRNA and protein concentrations. They are caused by transcriptional and translational feedback loops of the so-called clock-genes on their own expression (Fig. 1, for a review, see [12]). The heterodimer BMAL1:CLOCK activates the transcription of *period* (*per*), *cryptochrome* (*cry*) and *rev-erba* genes. After translation and posttranslational processes (e.g. phosphorylation, complex formation, nuclear translocation) PER2

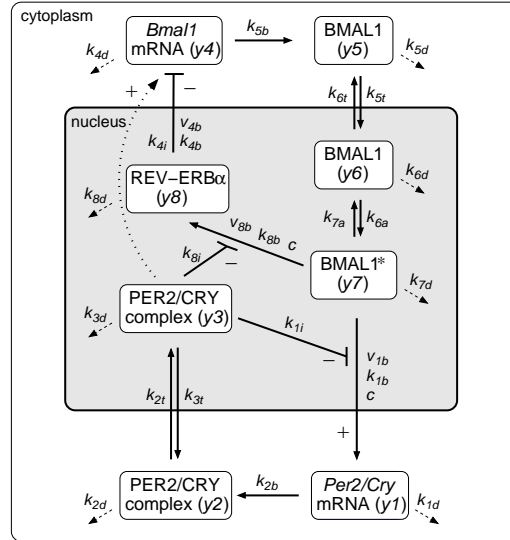


Figure 1: Model of the mammalian circadian oscillator: BMAL1* activates the transcription of *Per2* and *Cry* genes and the synthesis of REV-ERB α resulting in an increase of *Per2/Cry* mRNA (y_1) and REV-ERB α (y_8). As the levels of PER2 and CRY increase, they form a complex (y_2), which is transported to the nucleus. The nuclear PER2/CRY complex (y_3) inhibits *Per2/Cry* transcription and REV-ERB α synthesis. As REV-ERB α represses the *Bmal1* transcription, its decrease leads to an increase of *Bmal1* mRNA (y_4) and protein (y_5). Nuclear BMAL1 (y_6) in its active form (BMAL1*, y_7) restarts transcription of *Per2* and *Cry* genes. Dashed arrows represent degradation of mRNAs and proteins. The activation of *Bmal1* transcription via PER2/CRY as assumed in a previous model without REV-ERB α [2] is indicated by a dotted arrow. Parameters of the reaction kinetics are given in Table 1.

and CRYs inhibit their own transcription; this corresponds to a negative feedback loop. In addition, PER2 has been shown to increase the transcription of *Bmal1*; it thereby increases its own rate of transcription and constitutes a positive feedback loop. More recently, REV-ERB α was found to repress the transcription of *Bmal1* [11]. Thus, BMAL1 indirectly inhibits its own transcription in another negative feedback loop. The activation of *Bmal1* transcription by PER2 is presumably via inhibition of *Rev-erba* transcription.

For the development of the current view of the circadian system, clock-gene mutants have been used as a valuable tool. Their phenotypes are diverse: While, e.g. the *rev-erba*^{-/-} mice show a circadian phenotype very similar to that of wildtype mice [11], *Bmal1*^{-/-} mutant mice are arrhythmic [3]. *Cry1*^{-/-} mutant mice display a decreased circadian period, whereas the period in *Cry2*^{-/-} mutant mice is increased [18, 19].

In this work, we extend a previous mathematical model of the circadian oscillator [2], that already describes the negative feedback of PER2 and CRY and the positive feedback of PER2 on its own transcription by explicitly including REV-ERB α . In this extended model we additionally consider the negative feedback of *Bmal1* on its own transcription via REV-ERB α . With an appropriate set of parameters, we obtain oscillations with the correct period and reasonable phases between the concentration peaks of several clock components. Parameter variations are used to simulate clock-gene mutations in the model. Parameter changes corresponding to the arrhythmic *Per2*^{Brdm1} mice and the *Bmal1*^{-/-} mice destroy oscillations. With a parameter variation that reflects the *Rev-erba*^{-/-} mutation, the oscillations are sustained. Assuming a different inhibitory strength of *Cry1* and *Cry2*, the model can account for the decrease and increase of the period in the corresponding knock-out mutants. The agreement with the experimental data and the simple structure of the model make it a

valuable tool to elucidate the function of the different feedback loops.

2 Method and Results

2.1 Construction of the Model

In this paper, we extend a model of 7 differential equations that describes the negative feedback of PER2/CRY on its own transcription and the indirect positive feedback via *Bmal1* [2]. For reasons of simplicity the monomeric proteins and their phosphorylation are not modeled explicitly; *Per2* and *Cry* are represented by the same variables describing the mRNA and the protein. *Per1* which presumably is more involved in light input than in the core mechanism of the clock is not considered; neither is *Per3* that seems to have no obvious circadian function. In this previous model, *Rev-erba* is not considered explicitly and the negative feedback of BMAL1 on its own expression via REV-ERB α is not accounted for.

Here, we include an additional variable for the REV-ERB α protein. Only one additional variable is needed if we consider the turnover of *REV-ERB α* to be comparatively large and the translation rate to be proportional to the mRNA concentration. Direct activation of PER2 on *Bmal1* transcription is possible, but an indirect action via inhibiting *Rev-erba* transcription is very likely. In this case, the synthesis of REV-ERB α increases with a rising amount of BMAL1 and decreases with an increase of PER2/CRY concentration. The transcription of *Bmal1* is decreased by REV-ERB α .

The model shown in Fig. 1 is described by the following set of differential equations:

$$\frac{dy_1}{dt} = \frac{v_{1b}(y_7^s + c)}{k_{1b}^s(1 + (y_3^p/k_{1i}^p)) + (y_7^s + c)} - k_{1d}y_1 \quad (1)$$

$$\frac{dy_2}{dt} = k_{2b}y_1^q - k_{2t}y_2 + k_{3t}y_3 - k_{2d}y_2 \quad (2)$$

$$\frac{dy_3}{dt} = k_{2t}y_2 - k_{3t}y_3 - k_{3d}y_3 \quad (3)$$

$$\frac{dy_4}{dt} = \frac{v_{4b}}{k_{4b} + (y_8^u/k_{4i}^u)} - k_{4d}y_4 \quad (4)$$

$$\frac{dy_5}{dt} = k_{5b}y_4 - k_{5t}y_5 + k_{6t}y_6 - k_{5d}y_5 \quad (5)$$

$$\frac{dy_6}{dt} = k_{5t}y_5 - k_{6t}y_6 - k_{6a}y_6 + k_{7a}y_7 - k_{6d}y_6 \quad (6)$$

$$\frac{dy_7}{dt} = k_{6a}y_6 - k_{7a}y_7 - k_{7d}y_7 \quad (7)$$

$$\frac{dy_8}{dt} = \frac{v_{8b}(y_7^r + c)}{k_{8b}^r(1 + (y_3^v/k_{8i}^v)) + (y_7^r + c)} - k_{8d}y_8 \quad (8)$$

Here, y_1 represents *Per2* and *Cry* mRNA concentrations, y_2 and y_3 describe the concentrations of the PER2/CRY complex in the cytoplasm and the PER2/CRY complex in the nucleus, respectively. The variable y_4 represents the concentration of *Bmal1* mRNA, y_5 of cytoplasmatic BMAL1 protein and y_6 of BMAL1 protein in the nucleus. The variable y_7 describes the concentration of a transcriptionally active form BMAL1*, which can be understood as a complex with CLOCK and/or as phosphorylated BMAL1. The concentration of REV-ERB α is given by y_8 .

To keep the model as simple as possible, most reactions are modeled by linear kinetics. Nonlinearities appear in the terms that control transcription of y_1 , y_4 and y_8 and due to complex formation (Eq. 2).

2.2 Circadian Oscillations

As the rate constants of the molecular processes in the circadian oscillator are not known, we used the oscillations themselves to fit the model to the experiment. Criteria were the existence of oscillations with a period of about 24 hours, the phase relation of the oscillator components and the response of the system towards parameter variations. Table 1 shows the reference set of parameters used for all further simulations if nothing else is mentioned. All parameters are given without units throughout the text for ease of reading.

Table 1: Parameter values.

Parameter	Value	Description
v_{1b}	18 nMhr ⁻¹	Maximal rate of <i>Per2/Cry</i> transcription
k_{1b}	1 nM	Michaelis constant of <i>Per2/Cry</i> transcription
k_{1i}	0.65 nM	Inhibition constant of <i>Per2/Cry</i> transcription
c	0.001 nM	Concentration of constitutive BMAL1*
p	8	Hill coefficient of inhibition of <i>Per2/Cry</i> transcription
s	5	Hill coefficient of activation of <i>Per2/Cry</i> transcription
k_{1d}	0.12 hr ⁻¹	Degradation rate of <i>Per2/Cry</i> mRNA
k_{2b}	0.3 nM ⁻¹ hr ⁻¹	Complex formation rate of PER2/CRY
q	2	Number of PER2/CRY complex forming subunits
k_{2d}	0.07 hr ⁻¹	Degradation rate of the cytoplasmatic PER2/CRY
k_{2t}	0.24 hr ⁻¹	Nuclear import rate of the PER2/CRY complex
k_{3t}	0.02 hr ⁻¹	Nuclear export rate of the PER2/CRY complex
k_{3d}	0.12 hr ⁻¹	Degradation rate of the nuclear PER2/CRY complex
v_{4b}	3.0 nMhr ⁻¹	Maximal rate of <i>Bmal1</i> transcription
k_{4b}	1 nM	Michaelis constant of <i>Bmal1</i> transcription
k_{4i}	0.9 nM	Inhibition constant of <i>Bmal1</i> transcription
u	3	Hill coefficient of inhibition of <i>Bmal1</i> transcription
k_{4d}	1.8 hr ⁻¹	Degradation rate of <i>Bmal1</i> transcription
k_{5b}	0.14 hr ⁻¹	Translation rate of BMAL1
k_{5d}	0.03 hr ⁻¹	Degradation rate of cytoplasmatic BMAL1
k_{5t}	0.15 hr ⁻¹	Nuclear import rate of BMAL1
k_{6t}	0.06 hr ⁻¹	Nuclear export rate of BMAL1
k_{6d}	0.03 hr ⁻¹	Degradation rate of nuclear BMAL1
k_{6a}	0.03 hr ⁻¹	Activation rate of nuclear BMAL1
k_{7a}	0.003 hr ⁻¹	Deactivation rate of nuclear BMAL1
k_{7d}	0.02 hr ⁻¹	Degradation rate of activated nuclear BMAL1
v_{8b}	10.6 nMhr ⁻¹	Maximal rate of REV-ERB α synthesis
k_{8b}	1 nM	Michaelis constant of REV-ERB α synthesis
k_{8i}	1.1 nM	Inhibition constant of REV-ERB α synthesis
r	1	Hill coefficient of activation of REV-ERB α synthesis
v	2	Hill coefficient of inhibition of REV-ERB α synthesis
k_{8d}	1.5 hr ⁻¹	Degradation rate of REV-ERB α

Using these reference parameters, the concentration of all components are oscillating. The period of the oscillations is 23.4 hours and therefore lies within the circadian range (Fig. 2A). In Table 2 the phases of maximum concentration are given for various clock components in circadian time (1 period = 24 circadian hours). As a reference phase we use *Per2/Cry* mRNA that by definition peaks at circadian time 7 (CT7), all other phases are determined with respect to this reference.

All calculated phases are in reasonable agreement with measured phases. A delay of several circadian hours is observed between the maximum concentration of *Per2/Cry* mRNA and the peak concentration of nuclear PER2/CRY. Like in experiments, the BMAL1 concentration peak appears long before the *Per2/Cry* mRNA concentration peak. The REV-ERB α concentration oscillates antiphase with the nuclear PER2/CRY.

A reliable circadian oscillator should be robust against small variations in the reaction rates in order to function properly. We examined the robustness of the system towards parameter changes with respect to the circadian period. The parameters were increased or decreased systematically by 10%. The oscillations persisted in all cases and the period was quite stable: The largest change of the period (about 10%) was found by decreasing the Michaelis constant of *Per2/Cry* transcription, k_{1b} (data not shown). This implies that slight perturbations of the clock components will not destroy the clock function.

Table 2: Peaking time of various clock components.

Phase	Model	Experiment	Reference
<i>Per2/Cry</i> mRNA	CT7	CT6-10	[12]
PER2/CRY nuclear complex	CT14.4	CT12-14	[12]
<i>Bmal1</i> mRNA	CT15.8	CT15-18	[12]
BMAL1 protein	CT21.7	CT17-21	[16]
REV-ERB α	CT5.8	CT6-10	[11]

2.3 Clock-Gene Mutants

Most mutations of clock-genes have a more or less severe effect on the circadian phenotype, either resulting in a loss of circadian rhythmicity or in a change of the circadian period under constant environmental conditions. These mutations can be investigated in order to validate or reject and to improve the model. The *in silico* homolog of a clock-gene mutation is a change of a certain parameter. We investigated the effect of several parameter changes that correspond to experimental clock-gene mutants.

In experiment, the mutation of *Bmal1* leads to a complete loss of circadian rhythmicity: The behavior of mutated mice is arrhythmic, and the oscillations of clock-gene mRNA and protein concentrations disappear [3]. In the model we simulate this knock-out mutation by a removal of *Bmal1* transcription, i.e. $v_{4b} = 0$. Indeed, we observe a loss of oscillations under these conditions (Fig. 2B), the system reaches a stable steady state with low levels of *Per2/Cry* mRNA and protein. Low constant levels of *Per2* have also been found in experiments [3].

Unlike for the *Bmal1*^{-/-} mutant, only slight changes of the circadian phenotype are observed, if *Rev-erba* is mutated [11]. The oscillations remain stable with a period slightly shorter than in the wildtype mice. As done for the *Bmal1* mutant, the *Rev-erba* mutation is modeled by setting its transcription rate v_{8b} to 0. With the resulting parameter set, the oscillations of *Per2/Cry* mRNA and protein remain stable. At the same time, *Bmal1* mRNA and protein are not oscillating, but are expressed at constant high levels (Fig. 2C). This corresponds to the nearly constant, high expression levels of *Bmal1* mRNA, that have been found experimentally in *Rev-erba*^{-/-} mice [11].

In our model, *Per2*, *Cry1*, and *Cry2* are represented by the same variable, therefore a mutation of either of those genes cannot be simulated by a loss of transcription as done for *Bmal1* and *Rev-erba*. In these cases, we instead change the activities of the nuclear PER2/CRY complex, considering the specific effect of either mutation on the different activities.

It has been found that the level of *Bmal1* mRNA in mice that lack PER2 is rather low; this lead to

the idea of PER2 being an activator of *Bmal1* transcription [13]. As PER2 is a much weaker inhibitor of *Per2/Cry* transcription than the CRYs [8], we assume that the main function of PER2 is activation of *Bmal1* transcription. Most probably this activation is via inhibition of *Rev-erba* transcription. Therefore, to simulate the *Per2* mutation, we decrease the inhibitory strength of PER2/CRY on the synthesis of REV-ERB α (i.e. we increase k_{8i}). As a result the oscillations disappear in the system and the levels of *Bmal1* mRNA are low (Fig. 2D). These results are in agreement with the arrhythmicity observed in *Per2^{Brdm1}* mice that lack functional PER2. They show an arrhythmic activity pattern under constant conditions [20] and blunted circadian oscillations of mRNA [13].

As both *Crys* are inhibitors of *Per* and *Cry* transcription, we simulate their mutation by a decrease of the inhibitory strength of PER2/CRY on their own transcription (i.e., an increase of k_{1i}). Not much is known about the differential function of the two homologs *Cry1* and *Cry2*. However, surprisingly the null-mutation of either gene has the opposite effect on the period: While in the *Cry1^{-/-}* mutant mice the circadian period is shorter than in wildtype mice, the period is increased in the *Cry2^{-/-}* mutant mice [18, 19]. One discussed explanation for this phenomenon is a different inhibitory strength of the two homologs, *Cry2* being the weaker inhibitor [6]. The difference in the inhibitory strength is either due to different concentrations of CRY1 and CRY2 or due to their different effect on BMAL1/CLOCK. As the concentrations of CRY1 and CRY2 in liver differ not much [9], the second alternative is more likely. In our model, both alternatives correspond to a stronger decrease of the inhibitory strength for simulation of the *Cry1^{-/-}* mutation and a weaker decrease of the inhibitory strength in case of the *Cry2^{-/-}* mutation. In Fig. 3 the period is shown for varying inhibitory strength (variation of k_{1i}), the black dot indicating the reference parameter. A decrease of the inhibitory strength first increases the period and then decreases the period. This reflects the period changes observed in the *Cry2^{-/-}* and *Cry1^{-/-}* mutant mice, respectively. If the inhibitory strength is decreased even further in the model, the oscillations cease to exist. This corresponds to the loss of rhythmicity in *Cry1^{-/-}/Cry2^{-/-}* double mutant mice. Our results indicate, that a difference in the inhibitory strength can account for the different phenotype without assuming a different mechanism of the two homologs.

In summary, the phenotype of all investigated mutations can be reproduced in our model by appropriate parameter variations.

2.4 Constant Activation of *Bmal1* Transcription

The examination of the circadian period under different conditions is a fundamental tool to improve our understanding of the circadian oscillator. One experimental method is the overexpression of certain clock-genes. The level of expression can be varied by using different promoters and amounts of vector. Overexpression experiments have been done for *white collar (wc)* — the counterpart of *Bmal1* in *Neurospora*. Here, the period decreases with rising amount of both WC homologs [4]. In a rescue experiment in mice, rising concentrations of the transcriptional activator CLOCK (i.e. the partner of BMAL1 for transcriptional activation) lead to a shortening of the period [1]. To our knowledge, *Bmal1* has not been overexpressed with different concentrations, as this is rather difficult to achieve in mammals. As an alternative one could use an *in vitro* system to investigate the effect of different BMAL1 concentrations.

Here, we simulate the overexpression of *Bmal1* by reducing the inhibitory strength of REV-ERB α almost to zero ($k_{4i} = 1000$). The amount of *Bmal1* mRNA is varied by variation of v_{4b} . The system is not oscillating below a threshold of v_{4b} ($v_{4b} < 0.31$), above this threshold we observe first an increasing, than a decreasing period with rising *Bmal1* expression (Fig. 4, solid line). The decrease of the period near the reference point is related to the high Hill-coefficient $s = 5$ in Eq.1, that quantifies the impact of BMAL1 concentration on the *Per2/Cry* transcription. With a lower Hill-coefficient ($s = 1$, dashed line) the period increases with rising v_{4b} near the reference point.

These results indicate that by investigating the effect of different BMAL1 concentrations on the period one may find out how the *Per2/Cry* transcription is regulated by BMAL1. The comparison of

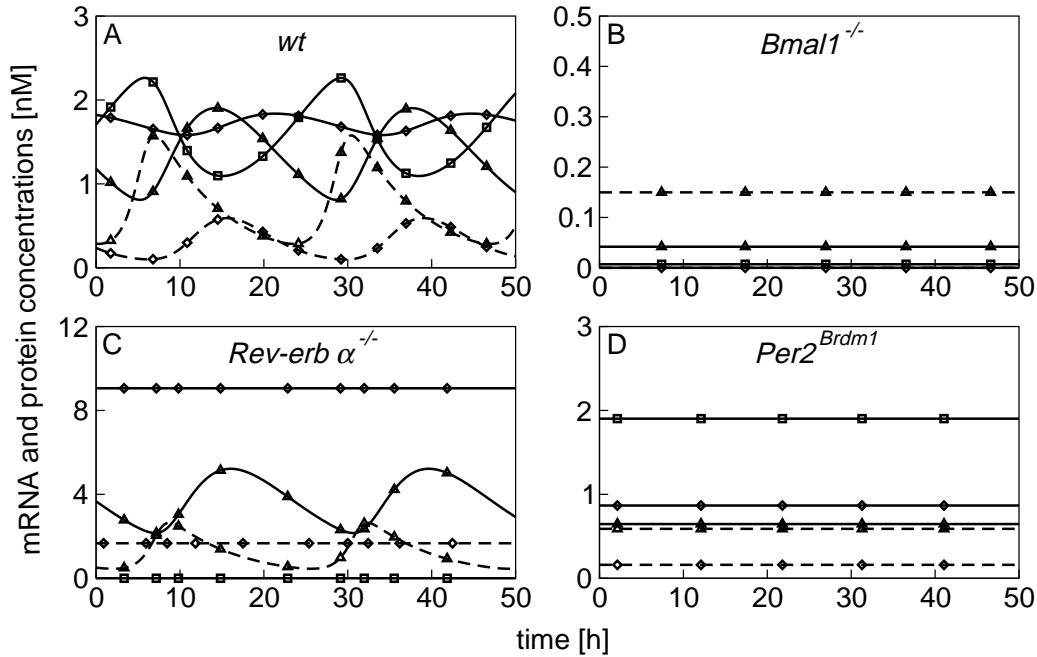


Figure 2: The model shows the correct dynamic behavior in simulations corresponding to wildtype and circadian mutants. *Per2/Cry* mRNA: dashed line, triangle; nuclear PER2/CRY: solid line, triangle; *Bmal1* mRNA: dashed line, diamond; BMAL1 total protein: solid line, diamond; REV-ERB α : solid line, square. A: Circadian oscillations obtained with the reference parameters given in Table 1. The period of the oscillations is 23.4 hours. Similar oscillations of clock-gene mRNA and protein concentrations have been found in wildtype mice. B: Without transcription of *Bmal1* ($v_{4b} = 0$) the system reaches a stable steady state. This corresponds to the arrhythmicity observed in *Bmal1*^{-/-} mutant mice. C: The oscillations persist without synthesis of REV-ERB α ($v_{8b} = 0$). In experiments, *Rev-erb α* ^{-/-} mice have been shown to have a rhythmic phenotype. D: The *Per2*^{Brdm1} mutation is modeled by a decreased inhibitory strength of PER2/CRY on REV-ERB α synthesis ($k_{8i} = 11$). This leads to a loss of oscillations as found experimentally.

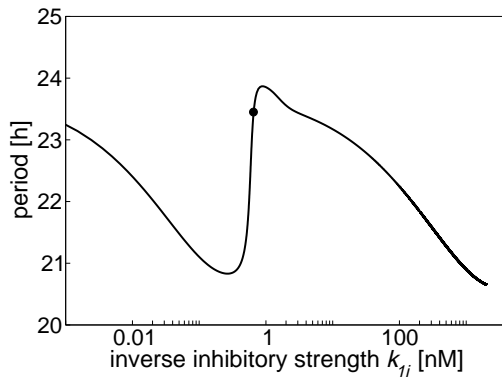


Figure 3: The opposite effect of $Cry1^{-/-}$ and $Cry2^{-/-}$ mutation on the period is reproduced in the model. The black circle marks the reference conditions corresponding to the wildtype. The $Cry1^{-/-}$ and $Cry2^{-/-}$ mutations are modeled by a decrease of the inhibitory strength of PER2/CRY on its own transcription (i.e. an increase of k_{ii}). With a slight decrease the period becomes longer, as has been shown for the $Cry2^{-/-}$ mutant mice. A strong decrease leads to a shorter circadian period as observed for the $Cry1^{-/-}$ mutant mice. With a further decrease of the inhibitory strength the oscillations disappear. The corresponding $Cry1^{-/-}/Cry2^{-/-}$ double mutant mice become arrhythmic.

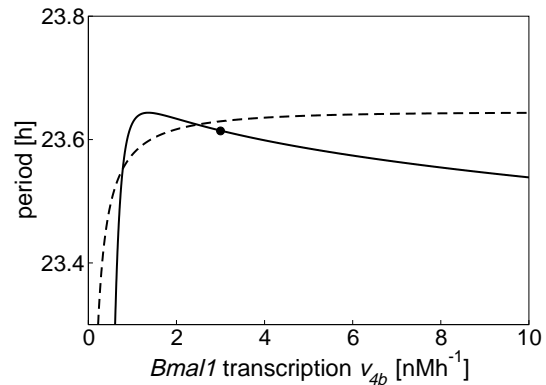


Figure 4: The period changes that occur with variation of the amount of BMAL1 depend on the Hill-coefficient s . We modeled the REV-ERB α independent overexpression of $Bmal1$ ($k_{4i} = 1000$) and varied the amount of $Bmal1$ mRNA and protein by changing v_{4b} . Around the reference point $v_{4b} = 3$ (black circle) the period decreases with rising $Bmal1$ transcription for $s = 5$ (solid line) and increases for $s = 1$ (dashed line). As the Hill-coefficient indicates the impact of BMAL1 on $Per2/Cry$ transcription, the period changes occurring in overexpression experiments give hints on this impact.

simulations with forthcoming experiments might reveal possible explanations of period changes.

3 Discussion

The discussed model of the circadian oscillator represents an extended version of a previous model with the additional clock-gene *Rev-erba* [2]. With this additional component the model represents better the biological mechanisms and accounts for phase delays between the nuclear PER2/CRY accumulation and the activation of *Bmal1* transcription. The model shows sustained oscillations with a circadian period and reasonable phases of the mRNA and protein concentrations. With this model we can reproduce the phenotype of several clock-gene mutations. Comparable results for the oscillations as well as for the mutations have been obtained using the model without *Rev-erba* [2]. Thus, essential features of the model are robust with respect to the incorporation of additional components.

A widely discussed question is why the circadian system consists of various interlocked feedback loops rather than a single delayed negative feedback loop, that would be sufficient to obtain oscillations [5]. With the previous model we were able to show that with the additional positive feedback we can account for the results of some non-intuitive double mutation experiments [2]. Here, we included an additional negative feedback in order to investigate its impact on the dynamics: BMAL1 is inhibiting its own expression via activation of *Rev-erba* transcription, which is an inhibitor of *Bmal1* transcription. First studies indicate that the robustness of the system towards parameter variations with respect to period and phases does not depend critically on this feedback. Other possible functions of the additional feedback are temperature compensation of the circadian oscillations and the regulation of input and output phases. In a recent work Leloup and Goldbeter show that due to this negative feedback oscillations with a different period can occur even if *Per2* is mutated [10]. Indeed, ultradian rhythms are observed in mice if the circadian control of activity is disturbed [7]. The molecular basis is not known. In our model we have not yet observed oscillations in the *Per2* mutant.

In our studies we found that the behavior of the system depends on the Hill-coefficients used (Fig. 4). A model of the circadian oscillator by Ueda *et al.* [17] can be simplified in a way, that it has the same topology as our model. However, it uses different kinetics and more variables. Therefore, a comparison of both models is well suited to examine the impact of those features on the dynamic behavior of the system. A comparison of several independent models will help to distinguish between characteristics of the specific model and the generic properties of the oscillator.

With this model we have a valuable tool at hand to decipher the functions of yet another negative feedback loop in the circadian oscillator. Due to the relatively simple structure of the model and the small number of variables the model is very well suited for this task.

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