MULTIPLE FEEDBACK LOOPS IN CIRCADIAN CYCLES: ROBUSTNESS AND ENTRAINMENT AS SELECTION CRITERIA

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ABSTRACT

This paper discusses the contribution of an additional feedback loop to the entrainment and the robustness of a circadian system. To quantify robustness, we perform a global analysis of the system's parameter space. We quantify the parameter region where the circuit displays an experimentally observed behavior, under entrainment. This global measure is comleted with a classification based on the phase response curve (PRC). For two models of circadian rhythms, we found that the one with two loops is more robust than the one with a single loop: the two-loop model shows better resilience to parameter perturbations and it has also a larger region where the PRC matches experimental PRCs of circadian oscillators.

1. INTRODUCTION

Circadian cycles have been widely studied in the recent years. The first model of a circadian rhythm was published about fifteen years ago [1] and includes only a single protein, Period (PER), that inhibits its own production. Since then, due to the discoveries of new key proteins, models of different complexity for several organisms have been published [2, 3, 4]. Even though the proteins differ from one organism to the other, most of the circadian oscillators are made of interlocked feedback loops that generally comprise negative regulators, but may also have additional positive feedbacks [5]. The generic mechanism is based on the production of several proteins and their post-translational modification (mostly through phosphorylation). Once modified, the proteins migrate to the nucleus (sometimes as a complex of different proteins) where they regulate their own expression or the expression of other proteins in the system.

Computational biologists have analyzed the robustness of circadian cycles in terms of local and global sensitivity. Broadly speaking, robustness is a property that allows a system to maintain its function despite external and internal perturbations. In this sense, circadian cycles have been postulated as system with high robustness: they are able to produce regular oscillations with a period of about 24 hours. Moreover, these systems have a very low sensitivity to temperature changes [6], molecular noise [7] and parameter variations [8].

Currently, it is widely believed that the specific structure of these biological networks is responsible for their dynamical behavior and their robust performance [9]. However, the actual mechanisms are still not clear. For example the purpose of the multiple feedback loops present in many circadian networks remains controversial [10]. For example, redundancy has been pushed forward as explanation for the emergence of these, often parallel, control mechanisms: Udea et al. showed that adding a feedback loop in a circadian clock enhances the robustness of the system to point mutations [6].

Surprisingly, most of the studies on robustness of circadian cycles have been performed on the autonomous oscillations, i.e. without any external entrainment [8]. They are therefore based on the properties of the system in a dark environment, whereas the main feature of circadian clocks is their ability to be entrained by the daily light/dark rhythm. In this research, we want to overcome this shortcoming and try to understand how the oscillator architecture influences the robustness of a system in relation to its entrainment properties.

We investigate this problem with two particular models of circadian clocks in *Drosophila*. As described above, the first published model of the circadian rhythm in *Drosophila* comprises only the protein PER that forms a single feedback loop [1]. The protein is going through two successive phosphorylation steps before entering the nucleus and inhibiting its own expression. These intermediate stages induce a delay in the feedback, a necessary condition for oscillations [11]. A second model followed, proposing an additional feedback loop through the action of the Timeless (TIM) protein. TIM acts in parallel to PER but its degradation is enhanced by light allowing the system to be entrained [12]. Further experimental studies have found the existence of another feedback loop with the Cycle protein and the Clock protein [2]. Other recently published models propose several new components [4], but we will focus on the first two systems which are generic models of moderate complexity for circadian clocks and are composed of either one [1] or two feedback loops [12] (see Fig. 1).

For this study, we combine a global analysis of the parameter space that quantify the robustness of biochemical oscillator models [13] and a further classification based on the phase response curve (PRC) analysis. The global approach identifies the ‘viable’ region of the high-dimensional parameter space where both models display the experimentally observed behavior under entrainment (period of the cycle and phase of the peaks). This global measure is followed by the PRC analysis that qualitatively assesses the different viable parameter sets. PRCs have proven to be a useful tool to study the input-output properties of oscillators [14]. It tabulates the phase shift at steady state that results from a particular input as a function of the phase at which this input is received. We perform this two-stage analysis on the models with one and two loops and compare the results.

2. RESULTS

2.1. Models

For the one-loop model, we use the model and its parameters as published by Goldbeter [1]. The two-loop model published by Leloup and Goldbeter [12] was selected as a comparison. As no entrainment was implemented for the one-loop model, we changed this model by adding a light-modulation of the degradation of the PER protein as it is the case for the TIM protein in the two-loop model (see Fig. 1). The parameters are the same as the ones used in the original paper. For this latter model, we performed two sampling procedures, where the restriction on the parameter symmetry between the two loops was released for one of the two sampling procedures. To this end, we obtain three models: one loop, two symmetric loops and two asymmetric loops. For all models, we use three different operating conditions that form a hierarchy: the sampling is done (1) under normal entrainment, then refined (2) by studying a few cycles after interruption of the entrainment and finally (3) in dark conditions (free-run).

2.2. Global analysis

To sample the parameter space for both models, we use the algorithm developed by Hafner et al. [13]. The criteria chosen for a parameter set to be viable are consistent with the experimental findings on the *Drosophila* clock. More specifically, we select parameter sets for which the models, under entrainment (light acts on the system from hour 0 to 12, i.e. ZT0 to ZT12), show the following criteria [15]:

- the oscillations are stable with a period of 24 hours (with a small margin of 0.05h to account for numerical errors) with an entrainment of 24 hours period;
- the relative amplitudes $\left(\frac{\max-\min}{\max}\right)$ of *per* mRNA and the nuclear complex (nPER or nTIM-PER) concentrations are of more than 60%;

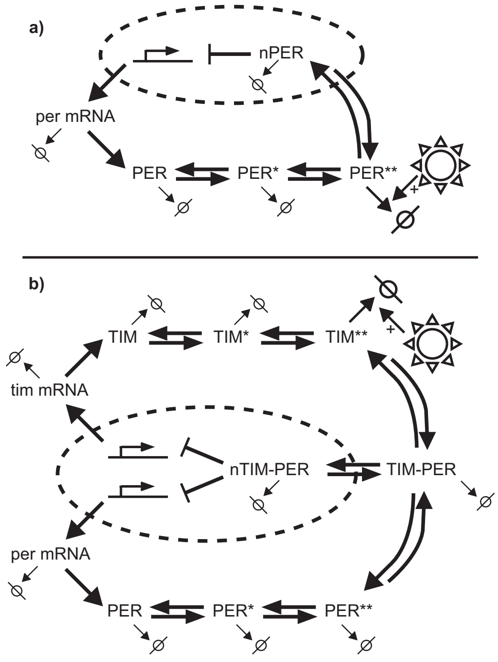


Figure 1. The one-loop (a) and the two-loop (b) models used for this study, adapted from [1] and, respectively, [12]. PER (and TIM) have two successive phosphorylation steps (indicated as *). PER** or the TIM-PER complex enters the nucleus (dashed circle) and inhibits gene expression (nPER and nTIM-PER stands for nuclear PER or nuclear complex). The light periodically enhances the degradation of PER** or, respectively, TIM**.

- the *per* mRNA concentration peaks during the early night (between ZT12 and ZT19 hours);
- the nuclear component concentration peaks in the late night (between ZT18 and ZT3 hours) and is with a delay of 4.5 to 10 hours after the peak of *per* mRNA;
- the *tim* mRNA and *per* mRNA concentrations peak within less than two hours difference (for two-loop models).

The sampling procedure is performed over a large range of four orders of magnitude around the original parameter set. With this broad sampling, we suggest that the computed characteristics are inherent in the structure and not in the parametrization of the models.

The results of the global analysis show that the model with two loops is more resilient to parameter perturbations: the region of the parameter space where the model is properly entrained by an external signal is larger. The model with one loop has on average around two and a half orders of magnitude (2.43 ± 0.02) of freedom per parameter to fulfill the defined criteria. The symmetric and asymmetric two-loop models have respectively 7.4% and 14.0% more freedom (normalized volume of 2.61 ± 0.03 and 2.77 ± 0.01) reflecting a higher global parameter robustness. The error is the standard deviation of three different simulations. More on the error of the method is discussed in [13].

We further refine our analysis by checking how the oscillations behave when the entrainment is released. This is performed in two stages. First we check if the system is able to maintain proper oscillations for three cycles in the dark (without entrainment). We use the same criteria as above but with a range 10% larger for the phase of the peaks and a period in the interval [21.6h, 26.4h]. The hierarchy of results for the three models remains the same, but interestingly the one-loop model shows a 5.3% decrease (to a value of 2.30 ± 0.02) whereas both two-loop models have only 2.8% and respectively 2.6% less freedom (values of 2.53 ± 0.05 and 2.70 ± 0.01).

We pursue the refinement by selecting the parameter sets that have a proper free-run behavior as observed experimentally. For this stage the criteria about the absolute phases are not anymore significant and only the ones for the period (10% around 24 hours), the relative amplitude of some components (more than 60%) and the relative phases (between peaks of mRNA and nuclear component concentrations) remain. For this last analysis, the difference is even stronger with the average parameter variation dropping to 2.12 ± 0.03 for the one-loop model (-12.0% compared to the case with entrainment). On the contrary, the two-loop models show both a decrease of 5.1% to a value of 2.46 ± 0.05 and 2.63 ± 0.02, respectively.

Breaking the symmetry of the parameters of the two loops increases only slightly the viable parameter space. The little difference could be explained by the low constraints on the phosphorylation steps. In the asymmetric model, there are two times more parameters for phosphorylation (for the PER and the TIM loops) and being the least constrained parameters, they increase the average value, when changing independently. More significant is the difference in the decrease of the normalized viable volume due to the refinement (relaxation and free-run operating conditions). This reduction is two times larger for the one-loop models than for the model with two loops. As both two-loop models have the same decrease we can argue that the advantage of the two-loop models in terms of robustness for an entrained system comes from the structure and not from an artifact due to the number of parameters.

2.3. Classification based on the PRC

The qualitative analysis is based on the phase response curve. The PRC mesures the positive (or negative) time shift in the phase (usually in hours) that results form an input given at a specific phase of the cycle. Experimental PRCs of *Drosophila* circadian clock (a diurnal organism) exhibit delay phase shifts for light pulses in the early subjective night, advanced phase shifts in the late subjective night and little phase shifts during the subjective day (this region is called the dead zone) [16]. This specific PRC profile allows the system to be entrained at the correct phase with a periodic light signal. We use the PRC to define qualitative measures of the entrainment and use it as a discriminative criterion for model selection. More specifically, we focus on the following aspects: positions of the extrema and occurrence of a dead zone.

First we sample again the parameter space for sets that fulfill the free-run criteria. The measure of the viable volume shows an even stronger advantage for the two-loop models: the symmetric and asymmetric two-loop models have a normalized viable volume of 2.57 ± 0.03 and, respectively, 2.72 ± 0.01 whereas the value of the one-loop model is of 2.24 ± 0.04 only.

According to the analysis of the PRC for the different viable points in the parameter space, we define three classes of PRCs based on the position of the maximum and minimum and the existence of a dead zone (at least 9.6 circadian hours at a value inside a range 10 times smaller than $[\min(PRC), \max(PRC)]$). In the first class, parameter sets exhibit PRCs with a minimum followed by a maximum and then a dead zone (in accordance with experimental PRCs), the second class has PRCs with an inversion in the order of the minimum and maximum and, finally, the parameter sets of the third class exhibit PRCs without any dead zone.

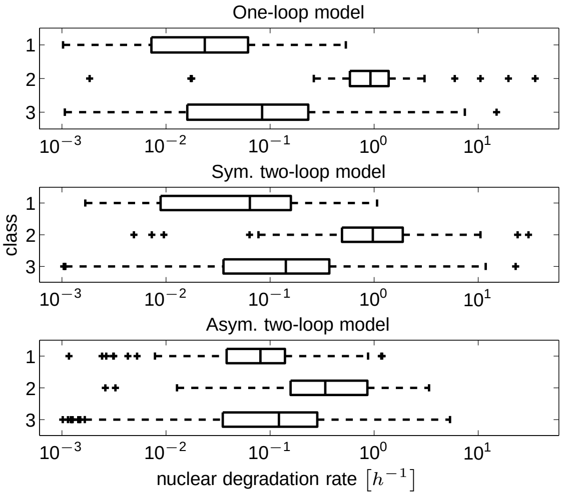
With this classification the normalized volume of parameter sets with PRCs of class 1 is reduced by 15.3% (value of 1.90 ± 0.06) in the case of the one-loop model. On the contrary, for both two-loop models, the volume where the parameter sets show a PRC comparable to the experimental ones remains higher: the viable volume decreases by 9.5% for the symmetric two-loop model and by 9.2% for the asymmetric one (values of 2.33 ± 0.06 and, respectively, 2.47 ± 0.04). This local analysis emphasizes the advantage of an additional feedback loop for the entrainment properties.

A closer look at the distribution of the three classes along the different parameters shows a clear bias toward low values of the parameters controlling the concentration of the nuclear component. More specifically, for all three models, the degradation rate of the component in the nucleus (nPER or nTIM-PER) is significantly lower for parameters in class 1 (Wilcoxon's rank sum test with $p < 0.05$, see Fig. 2). Except for the asymmetric two-loop model, the translocation rates (in or out of the nucleus) are also significantly lower in class 1 (p-value < 0.05). These differences are stronger for the one-loop model. Other parameters have no significant difference with respect to the three classes.

3. CONCLUSION

Taking into account the entrainment for the analysis of robustness is a logical step when studying circadian oscillators. Our work suggests that the additional loop enhances the robustness of the entrained system.

First, the global analysis reveals two benefical features of an additional feedback loop: (1) a larger region of the parameter space shows viable results and (2) a larger fraction of it maintains regular oscillations after the entrainment is stopped. This is a critical property for a circadian clock upon which evolutionary selection pressure


One-loop model

Sym. two-loop model

Asym. two-loop model

Figure 2. Boxplot of the nuclear degradation rate for the three classes of PRCs for the viable parameter sets in the different models. In all models, the average of the distribution for the class 1 is significantly lower than other classes however this difference is more important for the one-loop model.

may act. We also observe that the asymmetry introduced between the two loops does not change the results through the refinement: the robustness comes from the architecture (two-loops) and not from the particular parametrization of the models.

Second, with the classification based on the PRC, we can investigate what are the critical parameters that influence the entrainment properties. Our global sampling helps to understand the interactions between specific rates and PRC profiles that are consistent with experimental data. The results show that the rate of the reactions (degradation and translocation) controlling the concentration of the inhibitory component (nPER or nTIM-PER) in the nucleus can discriminate between the different PRC classes.

Interestingly, this part of the models, that can be considered as the most sensitive one, is strongly simplified in comparison to the most recent models [2, 4]. In fact, the inhibition by the TIM-PER complex is mediated through another complex Cycle-Clock, also controlled by positive and negative feedbacks. An interesting perspective is thus to study how these supplementary feedback loops influence the robustness and the entrainment properties and if they can overcome the weaknesses found by our research on the simpler models.

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