**Mathematical Analysis of Robustness of Oscillations in Models**

**of the Mammalian Circadian Clock**

**Short title: Robustness of Oscillations in Models of the Mammalian Circadian Clock**

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**ABSTRACT**

Circadian rhythms in a wide range of organisms are mediated by molecular mechanisms based on transcription-translation feedback. In this paper, we use bifurcation theory to explore mathematical models of genetic oscillators, based on Kim & Forger’s interpretation of the circadian clock in mammals. At the core of their models is a negative feedback loop whereby PER2 protein binds to and inhibits its transcriptional activator, BMAL1. For oscillations to occur, the dissociation constant of the PER2:BMAL1 complex, , must be ≤ 0.04 nM, which is at least 250-fold smaller than a reasonable expectation () for this protein complex. We relax this constraint by two modifications to Kim & Forger’s ‘single negative feedback’ (SNF) model: first, by introducing a multistep reaction chain for posttranslational phosphorylations of PER2, and second, by replacing the first-order rate law for degradation of PER2 in the nucleus by a Michaelis-Menten rate law. These modifications increase the maximum allowable to ~1 nM. In a third modification, we consider an alternative rate law for gene transcription to resolve an unrealistically large rate of *Per2* transcription at very low concentrations of BMAL1. Additionally, we studied extensions of the SNF model to include a second negative feedback loop (involving REV-ERBα) and a supplementary positive feedback loop (involving RORβ). We find that all three models are similarly robust in terms of accounting for circadian rhythms (~24 h period) with ≈ 1 nM. Contrary to Kim & Forger’s observations of these extended models, we find that, with our modifications, the supplementary positive feedback loop makes the oscillations more robust than observed in the models with one or two negative feedback loops. Our results provide testable predictions for future experimental studies. [ ~280 words; limit = 300 ]

**AUTHOR SUMMARY**

The circadian rhythm aligns bodily functions to the day/night cycle and is important for our health. The rhythm originates from an intracellular, molecular clock mechanism that mediates rhythmic gene expression. It is long understood that transcriptional negative feedback with sufficient time delay is key to generating circadian oscillations. However, some of the most widely cited mathematical models for the circadian clock suffer from problems of parameter ‘fragilities’. That is, sustained oscillations are possible only for physically unrealistic parameter values. A recent model by Kim and Forger nicely incorporates the inhibitory binding of PER2, a key clock protein, to its transcription activator BMAL1, but oscillations in their model require a binding affinity between PER2 and BMAL1 that is orders of magnitude larger than observed binding affinities of protein complexes. To rectify this problem, we make several physiologically credible modifications to the Kim-Forger model, which allow oscillations to occur with more realistic binding affinities. The modified model is further extended to explore the potential roles of supplementary feedback loops in the mammalian clock mechanism. Ultimately, accurate models of the circadian clock will provide better predictive tools for chronotherapy and chrono-pharmacology studies. [ ~190 words; recommended 150 – 200 words ]

**INTRODUCTION**

Most organisms experience perpetual day/night cycles and need to synchronize their physiological functions with this potent external driving rhythm of light and temperature (1). Endogenous circadian rhythms meet this demand. These autonomous clock-like rhythms are driven by molecular mechanisms that generate oscillations of ~24 h period through negative feedback on gene expression (1-3). Although the genes and proteins constituting the circadian clocks in animals, plants and fungi are quite different, their essential interactions are remarkably similar. In all cases, the clock mechanism features a ‘core’ negative feedback loop: *A activates B activates C inhibits A*. In mammals, this loop consists of transcriptional regulation involving six genes: *Per1/2*, *Cry1/2*, *Bmal1*, and *Clock* (1-4). For convenience, in this work we drop the distinction between the homologous pairs of proteins PER1/2 and CRY1/2. In this mechanism (Figure 1), the heterodimeric transcription factor BMAL1:CLOCK activates *Per* transcription. *Per* mRNA is then translated in the cytoplasm, where PER protein binds with CRY and enters the nucleus. PER:CRY then binds with BMAL1:CLOCK to block its activation of *Per* transcription. PER:CRY’s cycle of production, nuclear entry, auto-inhibition, and subsequent degradation is widely acknowledged to be the source of circadian rhythmicity (5).

Over the past 50 years, many people have proposed mathematical models of circadian rhythms (5-11). In 1965, Brian Goodwin proposed a model of periodic enzyme synthesis based on negative feedback on gene expression (12, 13). At the time, Goodwin was not attending to circadian rhythms, because nothing was known then about the negative feedback of PER on its own synthesis. But his model was picked up later by Peter Ruoff (14-17) to explain many characteristic features of circadian rhythms. Recently, the core negative feedback loop of Goodwin’s model was extended with other feedback loops (as in Figure 1) to create more comprehensive and realistic models of circadian rhythms (18-20). While all of these models have much to commend, they suffer from a technical problem with the underlying ‘Goodwin oscillator’.

In his model of periodic enzyme synthesis, Goodwin assumed that the end-product of a metabolic pathway functioned as an inhibitor of expression of the gene encoding the first enzyme in the pathway. The inhibition was carried out by *p* molecules of end-product binding cooperatively to the transcription factor for the gene. In this scenario the rate of transcription is given by a Hill function, , where *Z* = concentration of end-product, *α*1 = maximum rate of transcription, and *K* = end-product concentration at half-maximal rate of transcription. In the Supplementary Materials, we define Goodwin’s model precisely, discuss its basic problem (for the model to oscillate, *p* must be greater than 8, which is unreasonable), and we describe two changes to Goodwin’s model that permit oscillations for smaller values of *p*.

One particularly interesting modification to Goodwin’s model was made by Jae Kyoung Kim and Daniel Forger (19), who replaced Goodwin’s view—of negative feedback by cooperative binding of a generic ‘repressor’ to a gene promoter—with their own model of stoichiometric binding of a repressor (PER:CRY) to an activator (BMAL1:CLOCK) of gene expression. Some characteristic features of the two models have been compared in (21, 22). In the following section, we describe the Kim-Forger model and show that, like Goodwin’s model, the Kim-Forger model also has a ‘parameter fragility’ problem. This analysis frames our proposals for more robust and realistic mathematical models of circadian clocks. Then, in the ‘Results and Discussion’ section, we present our results in detail.

**Kim & Forger’s Model**

In 2012, Kim and Forger (19) presented a model of the negative feedback loop controlling mammalian circadian rhythms (Figure 2a). The Kim-Forger (KF) ODEs are:

Kim-Forger SNF Model.

|  |  |  |
| --- | --- | --- |
|  |  | (1) |
|  |  | (2) |
|  |  | (3) |
|  | | (4) |

SNF stands for ‘single negative feedback’ (i.e., the core negative feedback loop involving PER:CRY inhibition of BMAL:CLOCK). As originally written, the KF model has three dynamical variables: = [*Per* mRNA], = [PER protein in the cytoplasm], = [PER protein in the nucleus] (i.e., PER:CRY in the nucleus). The BMAL:CLOCK transcription factor is denoted by A; is the total concentration of BMAL:CLOCK in the nucleus, and is the concentration of ‘free’ BMAL:CLOCK in the nucleus. (The ‘hat’ on each variable indicates a concentration in nanomole/liter; and is time in hours.) The factor is the probability that BMAL:CLOCK is not bound to its repressor, PER:CRY. By expressing the rate of transcription of *Per* mRNA to be proportional to , Kim & Forger are implicitly assuming that the total number of BMAL:CLOCK dimers is large enough to saturate the E-boxes on the *Per* genes, and that PER:CRY binds equally well to BMAL:CLOCK dimers that are either bound or not bound to an E-box (Supplementary Materials, ‘Deriving the rate laws for *PER* transcription’). Equation (4) is derived by solving the condition for equilibrium binding of BMAL:CLOCK (A) and PER:CRY (P) to form an inactive complex (C); namely,

The ’s and ’s are rate constants with appropriate units of concentration and time. It is commonplace in these models to assume that , because this condition is most conducive to oscillations.

First of all, we cast the equations on the left side of (1)‒(3) into dimensionless form on the right side by defining dimensionless concentrations, , and dimensionless time, . Furthermore, is the dimensionless rate of synthesis of *Per* mRNA (in a wild-type diploid cell). The other dimensionless parameters in Eqs. (1)‒(4) are = total concentration of BMAL in the nucleus. ~~In Tables S1 and S2 we provide definitions of the variables and kinetic constants in Kim & Forger’s SNF model.~~

In addition to the SNF model, Kim & Forger proposed two extended models, in which the core negative feedback loop involving PER and BMAL is supplemented with (either) an additional negative feedback from REV-ERB on transcription of the *Bmal1* gene (called the NNF model, Figure 2b) (or) an additional positive feedback from ROR on transcription of the *Bmal1* gene (called the PNF model, Figure 2c). Evidences for these interactions are found in references (23-27). The ODEs of Kim & Forger’s ‘NNF’ and ‘PNF’ models are presented in the Supplementary Materials~~, along with definitions of the variables and kinetic constants in Tables S1 and S2~~.

Notice that, in the SNF model, nonlinearity in the transcription term is due to tight stoichiometric binding between PER and BMAL, not (as in Goodwin’s equations) to cooperative participation of nuclear PER in the regulation of *PER* gene expression. Consequently, the SNF model circumvents the unreasonable cooperativity constraint (*p* > 8) of Goodwin’s model. (Don’t confuse the Hill exponent, *p*, in Goodwin’s model with the concentration of nuclear PER, *P*, in the KF model.)

While the SNF model appears to oscillate robustly and avoid Goodwin’s unrealistic constraint (*p* > 8), the SNF model has an unrealistic constraint of its own. To elaborate, we derive an equation for oscillations to arise in the SNF model.

***Locus of Hopf bifurcations in Kim & Forger’s SNF model.***

The condition for a Hopf bifurcation to arise in Eqs. (1)‒(4) is

|  |  |
| --- | --- |
|  | (5) |

where *P*ss, the steady-state solution of Eqs. (1)‒(4), satisfies the equation

|  |  |
| --- | --- |
|  | (6) |

Solving Eqs. (5) and (6) simultaneously, we derive

|  |  |
| --- | --- |
|  | (7) |

and from Eq. (6) we derive

|  |  |
| --- | --- |
|  | (8) |

Substituting (8) into (7), we find, after a little algebra, the condition for a Hopf bifurcation (HB):

|  |  |
| --- | --- |
|  | (9) |

where,

|  |  |
| --- | --- |
|  | (10) |

Solving the quadratic equation (9), we obtain as a function of , as plotted in Figure 3a. We must locate a wild-type (WT) cell somewhere within the oscillatory domain, far enough from the HB locus so that mutant cells overexpressing or under-expressing BMAL and PER are still rhythmic. To this end, we propose the following ‘five-point criterion’ for choosing the values of and for a WT cell: if the point locates a WT cell on the bifurcation diagram, then the points should also lie within the oscillatory domain. We introduce this constraint because: *Bmal1*+/− and *Clock*+/− cells, i.e., are rhythmic; *Per1*−/−*Per2*+/+ and *Per1*+/+*Per2*−/− cells, i.e., are rhythmic [Cermakian, 2001; Steinlechner, 2002; Pendergast, 2010]; mouse embryonic fibroblasts (MEFs) retain rhythmicity when co-overexpressing both *Bmal1*and *Clock* up to at least four-fold [Lee, 2011, Fig 3c]; and MEFs carrying extra copies of *Per1* or *Per2*, driven by a *Per2*-promoter, also retain rhythmicity [Lee, 2011, Fig 6]. The smallest values of that satisfy these requirements are ; see Figure 3a.

The oscillatory solution of the SNF model for this set of parameter values is illustrated in Figure 3b. The dimensionless period of oscillation is 3.8, which would correspond to a 24 h rhythm if *β* = 0.16 h−1. Nuclear PER, *P*(*t*), executes nearly sinusoidal oscillations around a mean value of 1000. The oscillations of *Per* mRNA, on the other hand, are slightly non-sinusoidal. This property of the model is not in contradiction to the evidently sinusoidal oscillations exhibited by luciferase ‘reporter’ genes driven by *Per2* promoters [Xu et al 2015] because those observations were made on populations of cells, which, in reality, cannot be perfectly synchronized. In Supplementary Figure S1 we show that the *Per* mRNA oscillations reported in Figure 3b, when averaged over a population of cells with a 10% dispersion of phase, appear perfectly sinusoidal.

The oscillations in Figure 3b require [PER]nuclear ≈ [BMAL]total , i.e., that the dissociation constant of the PER:BMAL complex is much smaller than the concentrations of the binding partners. To see why this is a problem, we must estimate by fitting [PER] and [BMAL] to experimental data.

***Estimation of* *from experimental data.***

We can estimate from the fact that there is a maximum of ~30,000 molecules of PER in a mammalian cell (28); hence, . In the SNF model, cytoplasmic PER is transported into the nucleus, so the rate at which PER molecules are lost from the cytoplasm, , must equal the rate at which PER molecules are gained in the nucleus, , assuming that there is not significant degradation of PER in the cytoplasm. (For an order-of-magnitude estimation, this is a reasonable simplifying assumption.) In this case, , and . From the simulation in Figure 3b, we find that at the peak of its oscillation, and from BioNumbers (29), we find that the volume of a typical mammalian cell nucleus is ~500 fL. Hence, 0.04 nM. In this case, *Â*T ≈ 40 nM, and the total number of BMAL molecules in a nucleus of volume ~500 fL would be ~12,000. The observed number of BMAL molecules in a cell is ~25,000 (28), which is not too far off, considering that some fraction of BMAL molecules may not localize to the nucleus or act as functional transcription factors.

Is  a reasonable estimate of the affinity of PER for BMAL? We expect the time constant for dissociation of the PER:BMAL complex to be on the order of minutes (i.e., ), because, if dissociation of the complex were slower, then the negative feedback of PER on BMAL would react sluggishly to changes in nuclear PER concentration, and also ‘sticky’ PER binding to BMAL would disrupt PER degradation. Furthermore, Eq. (4) implies equilibrium of PER-BMAL binding and would not hold with a much slower dissociation constant. With this estimate of the dissociation rate constant, the binding constant for the complex would have to be However, protein-protein binding rate constants are typically on the order of 106 M−1s−1 (30). So, we estimate that a physically realistic, minimum value for the dissociation constant of the PER:BMAL complex is , and we conclude that the dissociation constant we used in the SNF model is unrealistically small by at least 25-fold.

Fribourgh et al. (31) recently studied the docking of PER2:CRY1/2 to the core PAS domain of BMAL:CLOCK and measured . This estimate of is likely too large because the authors used only partial protein sequences. Since the true value of is likely between 1 and 100 nM, we will take as our benchmark.

To summarize, we find that circadian oscillations in KF’s original SNF model require a value of the PER:BMAL dissociation constant, ≈ 0.04 nM (or smaller), that is 250-fold less than a realistic estimate of = 10 nM, and 25-fold less than the minimum value, = 1 nM.

In this work we consider some realistic changes to the SNF model that increase the maximum permissible value of for oscillations. In the process, we come up with some other surprising reassessments of the KF model and its extensions.

**RESULTS and DISCUSSION**

**Longer Feedback Loop and Saturating PER Degradation Increase the Oscillatory Robustness of the Kim-Forger SNF Model.**

Our primary goal in modifying KF models is to alleviate the unreasonable constraint on , the dissociation constant of the PER:BMAL complex. To this end, we consider two changes to the SNF model: first, increasing the number of dynamical species in the PER-BMAL negative feedback loop, and second, introducing a Michaelis-Menten rate law for the degradation of nuclear PER. These same changes are known to increase the robustness of Goodwin’s model (as explained in the Supplementary Materials).

Longer feedback loop. In the SNF model, there is only one intermediate (Pc) between *Per* mRNA (M) and nuclear PER protein (P). However, the primary gene transcript must be processed and exported to the cytoplasm, where it is translated into nascent PER protein. PER protein must be phosphorylated multiple times (PER has 10-20 phosphorylation sites (32, 33)) and bound to CRY before it is transported into the nucleus. These steps insert a considerable time lag between *Per* gene transcription and the negative feedback on BMAL activity. To account for this time delay, we replace Pc in the SNF model by a sequence of species, P1, …, P*J* (note that the first few intermediates could be mRNA species), to obtain the modified ODEs:

|  |  |  |
| --- | --- | --- |
|  |  | (11) |
|  |  | (12) |
|  |  | (13) |
|  |  | (14) |

where *N* = *J*+2 is the total length of the negative feedback loop, and *A*free is still given by Eq. (4). This change lengthens the time between *Per* mRNA transcription and the negative feedback signal generated by nuclear PER and consequently increases the oscillatory potential of the negative feedback loop (34).

The longer feedback loop changes the condition for a Hopf bifurcation to arise in ODEs (11)-(14): the number ‘8’ on the left-hand-side of Eq. (5) is replaced by the number . Following the same derivation as before, we find that Eq. (9) determines *α* as a function of *A*T at the Hopf bifurcation, provided that

|  |  |
| --- | --- |
|  | (10') |

In Figure 4a we show that, as *N* (the length of the feedback loop) increases, the domain of oscillations in the (*α*, *A*T) parameter plane moves toward smaller values of *α* and *A*T. For example, for *N* = 8, applying the five-point criterion, we place the WT cell at *α* = 200, *A*T = 40; see Figure 4b. For this choice of parameter values, the oscillation is illustrated in Fig. 4c: period = 15.5, and the maximum value of *P*tot = 540. Following a similar argument as that for the original model with *N* = 3, we write Assuming the identities *α*3 = … = *αJ*+2 = *β*1 (for a simple phosphorylation chain) and (the conservation law for nuclear transport mentioned before, with replaced by ), we rewrite the relation above as . (When there is no chance of misunderstanding, we write *P*tot for .) So, in this case we might estimate that = 100 nM/540 ≈ 0.2 nM. However, ‘*P*tot’ includes *Per* mRNA species as well as PER protein species. So a better estimate of *P*tot might be ‘300’, in which case ≈ 0.33 nM, which is still 30-fold smaller than our estimate of 10 nM for the binding of PER to BMAL. Furthermore, in this case, we estimate = 13 nM (4000 molecules in a nucleus of volume 500 fL), which is perhaps too small compared to the observed number of ~25,000 BMAL molecules.

Saturating degradation of nuclear PER. PER is degraded by proteasomes after it is poly-ubiquitinated by the E3 ligase β-Trcp (35). Because the rate of this enzyme-mediated reaction likely saturates at large substrate concentration, it is reasonable to replace the linear kinetics for degradation of nuclear PER in Eq. (3) by a Michaelis-Menten rate law (35),

|  |  |  |
| --- | --- | --- |
|  |  | (14’) |

*β*max and *K*m are dimensionless parameters; in particular, , . This change also has the potential to increase the oscillatory robustness of the model. Intuitively, the upper limit to the rate of PER degradation introduced by the Michaelis-Menten rate law causes nuclear PER concentration to react sluggishly to changes in the rate of *Per* mRNA production, which is another sort of ‘lag’ in the negative feedback loop.

To keep track of these changes, we introduce the notation SNF(0DN), where D denotes the PER degradation rate law (L for linear or M for Michaelian), and N denotes the number of species in the negative feedback loop. For example, the original KF model is denoted SNF(0L3). The significance of the ‘0’ will become evident shortly.

For the case of ‘saturating degradation,’ we still scale all concentrations with respect to , but we can no longer derive a closed-form algebraic equation for the locus of Hopf bifurcations. Instead, for *N* = 8, we searched the four-dimensional parameter space (*α*, *A*T, 𝛽max, *K*m) for oscillations with the smallest value of *P*tot, subject to the constraints that *K*m > 1 and that the model gives a reasonable domain of oscillations in the (*α*, *A*T) plane (i.e., large enough to satisfy the five-point criterion). We found several different combinations of *β*max and *K*m that could satisfy these criteria with similar values of *P*tot, suggesting that the model is robust with respect to these criteria (see Supplementary Material). A typical combination is *β*max = 3.8 and *K*m = 1, shown in Figure 5a. The five-point criterion is satisfied for , and the oscillations for this case are shown in Figure 5b, for which *P*tot = 70; discounting for mRNA species, we estimate *P*tot = 50. Hence, = 100 nM/50 ≈ 2 nM and ≈ 40 nM (12,000 molecules in a nucleus of volume 500 fL). This estimate of the theoretical value of is now within our range of the probable experimental value, 1 nM < < 10 nM, and the estimate of the total number of BMAL molecules per nucleus is acceptable, considering our uncertainty about the localization of BMAL.

A disturbing property of this SNF(0M8) model is that oscillations persist even as *A*T → 0, which is clearly impossible because there can be no expression of the *Per* gene when BMAL concentration is zero. The problem, of course, is that the rate law for *Per* transcription (rate *A*free/*A*T) is valid only if BMAL saturates *Per* E-boxes, which clearly cannot be true as *A*T → 0. To get around this problem, we propose a revised rate law for *Per* gene transcription (see Supplementary Materials, ‘Deriving the rate laws for *Per* transcription’).

**A More Realistic Rate Law for *Per* Transcription Rate Law Does Not Increase the Robustness of the SNF Model.**

We propose to replace the KF expression for the rate of *Per* gene transcription (Rate Law 0) by a revised Rate Law 1 that is more realistic for small *A*T:

|  |  |  |
| --- | --- | --- |
| Rate Law 0: |  | (15-0) |
| Rate Law 1: |  | (15-1) |

For rate law 1, the maximum rate of transcription is , which depends on how strongly BMAL:CLOCK binds to the E-box, as characterized by the dimensionless dissociation constant ; and also, when *A*T becomes small, the transcription rate is proportional to *A*free/*K*A (not *A*free/*A*T). Rate law 0 applies to the case in which binding between PER:CRY and BMAL:CLOCK is independent of the binding between BMAL:CLOCK and E-box, and BMAL:CLOCK complexes saturate *Per* E-boxes. Rate law 1 relaxes the assumption of saturation of *Per* E-boxes by BMAL:CLOCK.

**Modified Kim-Forger SNF equations.** Taking all of the aforementioned changes into account, we have:

|  |  |  |
| --- | --- | --- |
|  |  | (16) |
|  |  | (17) |
|  |  | (18) |
|  |  | (19) |
|  |  | (20) |

where , and . (21)

In the notation SNF(TDN), T denotes the *Per* transcription rate law (0 or 1), and *N* = *J* + 2 = total length of the negative feedback loop.

Models of form SNF(1LN) can be analyzed exactly as SNF(0LN), and the condition for a Hopf bifurcation is Eq. (9), , where,

|  |  |
| --- | --- |
|  | (10'') |

Solving this quadratic equation for as a function of , we plot the locus of Hopf bifurcations for *N* = 3 and 8 in Figure 6a and b. Clearly, this change in rate law makes little difference in the robustness of oscillations for . As *K*A increases further, the bifurcation locus moves ‘up’ and *P*tot increases, so the estimated value of gets smaller.

For SNF(1MN) we have no closed-form algebraic equation for the locus of Hopf bifurcations, so as before, we set *N* = 8 and searched the five-dimensional parameter space (*α*, *A*T, *β*max, *K*m, *K*A) for oscillations with the smallest value of *P*tot, subject to the constraints

and that the amplitude of oscillation of *P*tot(*t*) be larger than 0.5, where . The amplitude constraint is to select for ‘robust’ oscillations. A summary of these calculations is provided in the Supplementary Material. Briefly, we found ~1000 parameter sets with max*P*tot ≈ 33 ± 5. Then we checked for parameter sets that satisfy the ‘five-point criterion’. Results of a typical parameter set (*β*max = 5, *K*m = 5.5 and *K*A = 20) are illustrated in Figure 7, for which max*P*tot = 75, larger than ‘33’ because the WT cell must be centered in the oscillatory domain of Figure 7a. Discounting *P*tot for mRNA species, we estimate max*P*tot = 40, Hence, = 2.5 nM and = 50 nM (~15,000 molecules of BMAL per nucleus). We conclude that, although rate law 1 is more accurate than rate law 0 for values of *A*T ≤ *K*A, it does not improve significantly on our estimates of and .

**Adding a Negative Feedback Loop Involving REV-ERB Does Not Increase the Robustness of Circadian Oscillations.**

Next, we explore Kim & Forger’s NNF model (see Supplementary Material), with modified rate laws for gene transcription. For the rates of transcription of *Per* and *Rev-erb* genes governed by BMAL:CLOCK binding to E-boxes, we use our Rate Law 1 (15-1). For the transcriptional repression of the *Bmal1* gene by REV-ERB (variable *V*), we replace Kim & Forger’s function *γ/V* by , where is the dissociation constant for REV-ERB binding to the promoter (RORE) of the *Bmal1* gene. This new rate law remedies an issue in KF’s original NNF model, for which the rate of synthesis of *Bmal1* mRNA → ∞ as *V*→0.

**Modified Kim-Forger NNF model.** Equations (16)-(21) plus

|  |  |  |
| --- | --- | --- |
|  |  | (22) |
|  |  | (23) |

The dimensional equations on the left-hand-side are cast into dimensionless form with the same definitions used in Eqs. (1)-(4), plus

To compare NNF to SNF, we must adopt some constraints on the new parameters. First of all, from Narumi et al. [REF], we find that the maximum number of REV-ERB molecules during the circadian rhythm in mouse cells (???) is 50,000. If all molecules are confined to a nucleus of 500 fL, then . For to be greater than, say, 10 nM, we will constrain *V*MAX so that . We continue to insist that the relative amplitude of *P*tot(*t*) be > 0.5, and, in addition, we constrain *A*MAX so that the relative amplitude of *A*(*t*) over an oscillation is > 0.2 (see, e.g., REF). Subject to these constraints, we search over the available parameter space

to minimize the objective function ; i.e., to maximize the value of .

A summary of these calculations is provided in the Supplementary Material. Briefly, we found ~1000 parameter sets with max*P*tot ≈ 50 ± 10. Again, after checking for parameter sets that satisfy the ‘five-point criterion’, we plot results of a typical parameter set (*β*max = 4, *K*m = 1.8, *K*A = 1.7, *V*MAX = 25 and *δ* = 0.16) in Figure 8. For the WT cell , max*P*tot = 55 and avg*A*T = 10. Discounting for mRNA species, max*P*tot = 30, = 3.3 nM and = 33 nM (~10,000 molecules of BMAL per nucleus). We conclude that NNF(1M8) is not more robust than SNF(1M8), nor does it improve our estimates of and .

**An Additional Positive Feedback Loop Involving ROR Increase the Robustness of Circadian Oscillations at a Cost.**

Next, we explore Kim & Forger’s PNF model, with similarly modified rate laws for gene transcription: Eq. (15-1) for the rates of transcription of *Per* and *Ror* genes, and for the rate of transcription of *Bmal1* by ROR (variable *R*). In the latter rate law, is the dissociation constant for ROR binding to the RORE promoter and ε is the fractional reduction in *Bmal1* expression when . This new rate law remedies an issue in KF’s original PNF model, for which the rate of *Bmal1* transcription does not behave appropriately as *R*→∞ or *R*→0.

**Modified Kim-Forger PNF model.** Equations (16)-(21) plus

|  |  |  |
| --- | --- | --- |
|  |  | (24) |
|  |  | (25) |

The dimensional equations on the left-hand-side are cast into dimensionless form with the same definitions used earlier, plus

To compare PNF to SNF, we adopt the following constraints. First of all, since maximum number of ROR molecules during the circadian rhythm in mouse cells (???) is 25,000 [Narumi et al. REF], we estimate that , and consequently we constrain *R*MAX so that . We continue to insist that the relative amplitudes of *P*tot(*t*) be > 0.5 and of *A*(*t*) be > 0.2. Subject to these constraints, we search over the available parameter space

to minimize the objective function ; i.e., to maximize the value of .

A summary of these calculations is provided in the Supplementary Material. Briefly, we found ~1000 parameter sets with max*P*tot ≈ 50 ± 10. Again, after checking for parameter sets that satisfy the ‘five-point criterion’, we plot results of a typical parameter set (*β*max = 8, *K*m = 10, *K*A = 2.5, *R*MAX = 70, *ε* = 0.0002 and *δ* = 25) in Figure 9. For the WT cell , max*P*tot = 21 and avg*A*T = 0.03. Discounting for mRNA species, max*P*tot = 14 and = 7 nM, which is quite reasonable, but ≈ 0.2 nM (i.e., 60 molecules of BMAL per nucleus), which is totally unreasonable. Clearly, the PNF(1M8) exhibits more robust oscillations than the SNF(1M8) and NNF(1M8) models and is consistent with ≈ 10 nM, but the oscillations are inconsistent with observed numbers of BMAL molecules in mammalian cells. The PNF(1M8) model oscillates with weaker binding of PER to BMAL because the predicted BMAL concentration in the nucleus is very small ( ≈ 0.03) and the maximum rate of expression of *Per* mRNA is very large (*α* ≈ 300). The oscillations, though ‘robust,’ are unlikely to represent the molecular mechanism in mouse cells.

ALTERNATE PNF(1M8)

A summary of these calculations is provided in the Supplementary Material. Briefly, we found ~1000 parameter sets with max*P*tot ≈ 50 ± 10. Again, after checking for parameter sets that satisfy the ‘five-point criterion’, we plot results of a typical parameter set (*β*max = 0.4, *K*m = 3, *K*A = 7, *R*MAX = 40, *ε* = 0.0001 and *δ* = 15) in Figure 9ALT. For the WT cell , max*P*tot = 4 and avg*A*T = 0.05. Discounting for mRNA species, max*P*tot = 2.5 and = 40 nM, which is quite reasonable, but ≈ 2 nM (i.e., 600 molecules of BMAL per nucleus), which is totally unreasonable. Clearly, the PNF(1M8) exhibits more robust oscillations than the SNF(1M8) and NNF(1M8) models and is consistent with ≈ 10 nM, but the oscillations are inconsistent with observed numbers of BMAL molecules in mammalian cells. The PNF(1M8) model oscillates with weaker binding of PER to BMAL because the predicted BMAL concentration in the nucleus is very small ( ≈ 0.05) and the maximum rate of expression of *Per* mRNA is very large (*α* ≈ 50). The oscillations, though ‘robust,’ are unlikely to represent the molecular mechanism in mouse cells.

STOPPED HERE. NEXT STEP IS TO LOOK AT REGION OF ‘CIRCADIAN OSCILLATIONS’ (PERIOD 22 – 26 H) IN THE OSCILLATORY DOMAINS OF 1M8 MODELS

**The NNF(1M8) Model Is Most Robust with Respect to Circadian Oscillations.**

Figure 5 redraws the bifurcation plots for the ‘1M8’ models in Figure 4c with colors to indicate oscillatory periods. To convert from dimensionless period *τ* to period in hours, we set of *β*1 = 5/6h, a value that yields a period of ~24 h for the models with their default parameter values (Table S4), in particular, *λ* = *φ* = 1 (Figure S1). For each model the oscillatory period varies over a characteristic range. For SNF(1M8) the period varies from 19 to 36 h, but the range of circadian rhythms (say, 22‒26 h) is quite limited. The NNF(1M8) model is more robust with respect to circadian periodicity. The PNF(1M8) model is much less robust, exhibiting very slow oscillations (up to 45 h) for modest overexpression of BMAL and PER. The broad distribution of oscillatory periods in the PNF model is a common feature of models that combine positive and negative feedback loops (34, 38). The NNF model has the nice property that oscillatory period is relatively insensitive to fold changes in BMAL expression, as observed in Supplementary Figure 1e of Xu et al. (39). All models predict that oscillatory period should be much more sensitive to fold-changes in PER expression than BMAL expression.

**CONCLUSION**

The Kim-Forger (KF) models of mammalian circadian rhythms (called SNF, NNF and PNF) are appealing in many respects, but they rely on an unrealistic requirement for robust oscillations, namely that the equilibrium dissociation constant of the PER:CRY::BMAL1:CLOCK complex must be < 0.04 nM, which is 250-fold smaller than a reasonable value for the PER:CRY::BMAL1:CLOCK complex, > 10 nM. This difficulty can be ameliorated (1) by lengthening the core negative feedback loop between *Per* mRNA transcription and PER:CRY inactivation of BMAL1:CLOCK (the transcription factor driving *PER* expression), and/or (2) by implementing a Michaelis-Menten rate law for the degradation of nuclear PER. The KF models were further modified by introducing an alternative rate law for BMAL1:CLOCK-mediated transcription of clock genes (*Per*, *Rev-erb* and *Ror*) to correct a problem at low expression of the *Bmal1* gene, and to provide more accurate rate laws for the effects of REV-ERB and ROR on *Bmal1* expression.

With these modifications, we find (Figure 7) that the SNF(1M8) model can exhibit oscillations for . From biophysical constraints, we estimate that , so the model constraint is not too far off from expectations. For , the SNF(1M8) model oscillates over a 14-fold range of total BMAL1 concentrations, 10 nM < *Â*T < 140 nM. For *Â*T = 30 nM, the corresponding number of BMAL1 molecules in a nucleus of volume 500 fL would be , which is about half the observed number (~24,000) of BMAL1 molecules in a mammalian cell (28). If the remaining BMAL1 molecules are dispersed through the cytoplasm of volume 5000 fL, the cytoplasmic concentration of BMAL1 would be about one-tenth the nuclear concentration, which is not unreasonable for a ‘nuclear’ protein such as BMAL1. Furthermore, the model focusses on BMAL1:CLOCK complexes that bind E-boxes to regulate gene expression. BMAL1 in this form may account for only a fraction of total BMAL1, if BMAL1, like PER2, undergoes multi-step post-translational modifications. Indeed, both BMAL1 and CLOCK are known to be phosphorylated at multiple sites, which affects their stability, nuclear accumulation, and activity of the BMAL1:CLOCK complex (40-42).

Replacing the linear rate law for nuclear PER degradation by a Michaelis-Menten rate law causes a dramatic change in the sensitivity of oscillation to the expression levels of *Per2* and *Bmal1* (compare Figures 4a and 5a). Models with linear PER degradation, e.g., SNF(0L8), predict that oscillations are possible over an ever broadening range of rates of *Per2* and *Bmal1* expression; e.g., (approximately) in Figure 4a. For a comparable model with Michaelis-Menten degradation, SNF(0M8), the oscillatory domain is bounded by maximal rates of expression: *α* < 45 and *A*T < 45 in Figure 5a. These contrasting results provide a testable prediction for future experimental exploration. By overexpressing *Per/Cry* genes and/or *Bmal1/Clock* genes under control of their normal (regulated) promoters (i.e., by manipulating *α* and *A*T), one could test whether nuclear PER degradation operates in a saturated (Michaelis-Menten) or unsaturated (linear) manner, which would be difficult to measure directly *in vivo*. In the same experiment, by measuring the dependence of oscillation period on *α* and *A*T (i.e., on fold-changes in expression of *Per*/*Cry* and *Bmal1*/*Clock*), one could test a second robust prediction of our models (Figure ??) that period length is much more sensitive to *α* than to *A*T.

The single negative feedback loop (SNF), whereby PER inhibits its own synthesis, can be supplemented with an auxiliary positive feedback from ROR (PNF) or a negative feedback from REV-ERB (NNF) on the synthesis of BMAL1. For their versions of these three models (0L3 versions), Kim & Forger observed a ‘robustness trend’ NNF > SNF > PNF, in terms of the size of the oscillatory domain in parameter space. For our versions of these models (1M8 versions), we observed different trends. If we define ‘robustness’ in terms of the size of the domains of circadian oscillations (22-26 h) in parameter space, then the trend is NNF > SNF ≈ PNF.

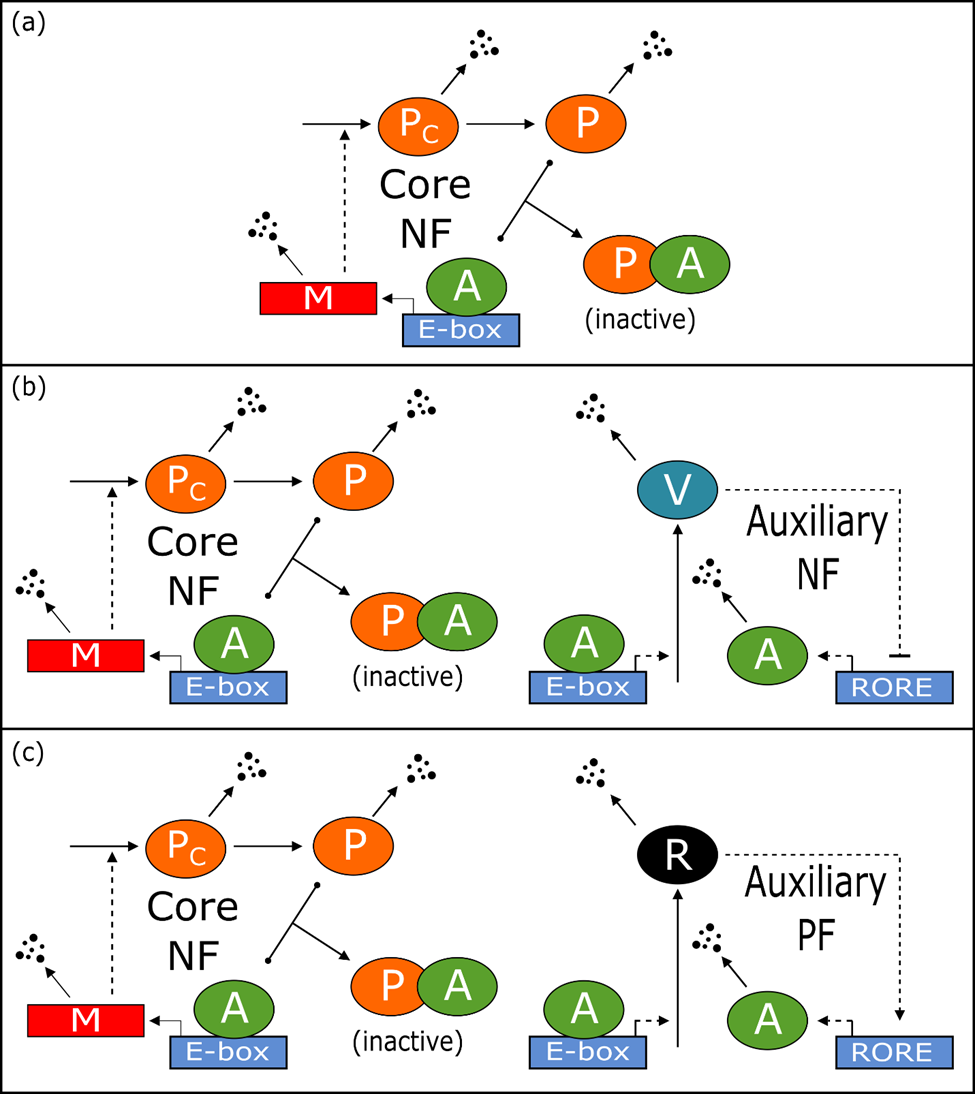
Our models could be employed in the future to explore other features of the mammalian circadian clock. For instance, following the lead of Kim and colleagues (43, 44), we could address our models to the circadian clock’s temperature-compensation and/or phase-shifting properties. Adding these key features may answer some remaining questions about the behaviors of these models. Another question that could be addressed with these models is the function of an anti-sense transcript of the PER2 gene (45). Furthermore, these models could be applied in chronopharmacology and chronotherapy studies (46). One such application would be modeling PER2’s interaction with the tumor suppressor protein p53 in stressed (e.g., DNA damage) cells compared to un-stressed cells (47, 48).

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**Figure 1.** Three major feedback loops regulate the mammalian circadian clock. The core negative feedback loop is between PER:CRY and BMAL1:CLOCK. The two sources of additional feedback are negative feedback between REV-ERB and BMAL1 and positive feedback between ROR and BMAL1. PER1/2, CRY1/2, and RORα/β are simplified to PER, CRY, and ROR, respectively. Solid lines indicate chemical reactions; the T-shaped reactions indicate reversible binding of proteins to form multicomponent complexes. Dashed lines indicate regulatory signals (positive regulation = barbed arrow, and negative regulation = blunt arrow).

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**Figure 2.** Wiring diagrams for the three Kim-Forger models: SNF **(a)**, NNF **(b)**, and PNF **(c)**. To simplify the models, several molecular species that do not contribute significantly to the feedback loops are not explicitly represented. For example, in the SNF loop, CLOCK and CRY are not shown. In the NNF and PNF loops, mRNAs encoding REV-ERB, ROR and BMALare not shown, nor are the cytoplasmic forms of these proteins. Solid and dashed lines indicate reactions and regulations, as in Figure 1.

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| **a** | **b** |

**Figure 3.** Oscillations in the SNF(0L3) model. **(a)** Hopf bifurcation curve given by Eqs. (9-10). ‘Five-point’ criterion: black circle, ‘homozygous diploid’ cell; white circles, heterozygous diploid cells; red circles, tetraploid cells. **(b)** Oscillations for homozygous diploid cells (*A*T = 1000, *α* = 20,000), Period = 3.8; *P*tot = 2650.

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| --- | --- |
| **a** | **b** |
| **c** | |
| **Figure 4.** SNF(0LN) models. **(a)** Loci of Hopf bifurcations for *N* = 3, 4, 6, 8. **(b)** Contour lines of constant *P*tot (expressed as ) for SNF(0L8) model. Circles mark the ‘five-point’ criterion, as in Figure 3a. **(c)** Simulation of WT cell, SNF(0L8) with *A*T = 40, *α* = 200. Period = 15.5, *P*tot = 540, . | |

|  |  |
| --- | --- |
| **a** | **b** |

**Figure 5.** SNF(0M8) model. **(a)** Bifurcation diagram for *K*d = 1, *V*max = 3.8, *K*m = 1. Five-point figure locates WT cell at the black dot. **(b)** Time-courses of *M*(*t*), *P*(t) and *P*tot(*t*) for WT cell: AT = 16, *α* = 20; Period = 30, *P*tot = 70.

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| **a** | **b** |
| **Figure 6.** Loci of Hopf bifurcations for **(a)** SNF(1L3) model and **(b)** SNF(1L8) model for *K*A = 1, 10 and 100. For comparison, *K*A = 0 (dashed lines) show contour for SNF(0LN). Rate law 1 makes little change to the oscillatory domain until *K*A > 10. | |

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**Figure 7.** SNF(1M8) model. **(a)** Bifurcation diagram for *β*max = 5, *K*m = 5.5, *K*A = 20. Five-point figure locates WT cell at the black dot. **(b)** Time-courses of *M*(*t*), *P*(t) and *P*tot(*t*) for WT cell: AT = 20, *α* = 50; Period = 27, *P*tot = 75.

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**Figure 8.** NNF(1M8) model. **(a)** Bifurcation diagram for *β*max = 4, *K*m = 1.8, *K*A = 1.7, *V*MAX = 25, *δ* = 0.16. Five-point figure locates WT cell at the black dot. **(b)** Time-courses of *A*T(*t*), *V*(*t*), *M*(*t*), *P*(t) and *P*tot(*t*) for WT cell: *A*MAX = 50, *α* = 16; Period = 24, *P*tot = 55.

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| --- | --- | --- | --- |
|  | |  |  |
| Ptot/7 (black), Pnuc (red), M (blue) | Ptot/7 (black), AT (red), R (purple) | | |
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**Figure 9.** PNF(1M8) model. **(a)** Bifurcation diagram for *β*max = 8, *K*m = 10, *K*A = 2.5, *R*MAX = 70, *ε* = 0.0002, *δ* = 25. Five-point figure locates WT cell at the black dot. **(b)** Time-courses of *M*(*t*), *P*(t) and *P*tot(*t*) for WT cell: *A*MAX = 0.1, *α* = 300. **(c)** Time-courses of *A*T(*t*), *R*(*t*), and *P*tot(*t*) for WT cell: *A*MAX = 0.1, *α* = 300; Period = 18, *P*tot = 22.

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| --- | --- | --- | --- |
|  | |  |  |
| Ptot/7 (black), Pnuc (red), M (blue) | Ptot/7 (black), AT (red), R (purple) | | |
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**Figure 9 ALTERNATE.** PNF(1M8) model. **(a)** Bifurcation diagram for *β*max = 0.4, *K*m = 3, *K*A = 7, *R*MAX = 40, *ε* = 0.0001, *δ* = 15. Five-point figure locates WT cell at the black dot. **(b)** Time-courses of *M*(*t*), *P*(t) and *P*tot(*t*) for WT cell: *A*MAX = 0.4, *α* = 50. **(c)** Time-courses of *A*T(*t*), *R*(*t*), and *P*tot(*t*) for WT cell: Period = 32, *P*tot = 4.

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