**SUPPLEMENTARY MATERIALS**

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

**of the Mammalian Circadian Clock**

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# Table S1. Definitions of the dynamical variables in the models\*.

|  |  |  |
| --- | --- | --- |
| **Dimensionless Variable** | **Definition** | **Physical Meaning** |
| *t* |  |  |
| *M* |  | *Per* mRNA |
| *Pi* |  | Cytoplasmic PER species |
| *P* |  | Nuclear PER |
| *A* |  | Nuclear BMAL |
| *R* |  | Nuclear ROR |
| *V* |  | Nuclear REV-ERB |

\*All ‘hatted’ variables and parameters carry units of concentration (nM) and time (h). We have assumed that all the first-order rate constants for loss of mRNA and cytoplasmic PER species are identical: .

# Table S2. Definitions of the dimensionless parameters in the models.

|  |  |  |
| --- | --- | --- |
| **Dimensionless Parameter** | **Definition** | **Meaning** |
| *K*d | 1 | Dissociation constant of the PER:BMAL complex |
| *K*A |  | Dissociation constant of the BMAL:Ebox complex |
| *K*m |  | Michaelis constant for the degradation of nuclear PER |
| *β*max |  | Maximum rate of degradation of nuclear PER |
| *α* |  | Maximum rate of transcription of *Per* gene |
| *A*T |  | Total BMAL concentration (bound + unbound) |
| *A*MAX |  | Maximum concentration of BMAL |
| *V*MAX |  | Maximum concentration of REV-ERB |
| *R*MAX |  | Maximum concentration of ROR |
| *δ* |  | Rate constant for turnover of BMAL, REV-ERB and ROR |

# Figure S1. …. ???????

# Goodwin’s model

To account for observations of periodic enzyme synthesis in bacteria [1], Brian Goodwin [2, 3] presented the following model for the periodic synthesis of an enzyme Y from its mRNA X, where mRNA synthesis is inhibited by a repressor Z that is the product of the catalytic action of Y:

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

In Eq. (1), the factor is the probability that the promoter region of the gene encoding X is not bound to Z, its repressor, and *α*1 is the maximum rate of synthesis of X by the gene. The other terms in these equations correspond to first-order rate laws for production and removal of X, Y and Z. In this tableau, Goodwin’s equations are written in two equivalent forms. On the left, the equations are written in terms of the original dimensional variables: concentrations *X*, *Y* and *Z* (nM) and time *T* (h); on the right, in terms of the ‘dimensionless’ variables , , , dimensionless time , and a dimensionless parameter . In deriving these dimensionless equations, we have assumed (as have all authors in the past) that , which serves to maximize the oscillatory potential of the model [4, 5]. In Goodwin’s version of a three-component negative-feedback loop, the repression of gene transcription by Z is modeled by a Hill function with exponent *p*. Underlying this function is the supposition that the gene encoding X is turned off when *p* molecules of Z bind cooperatively to its promoter region (or, equivalently, when *p* molecules of Z bind cooperatively to an activator of gene transcription and shut it off).

A problem with Goodwin’s model. J.S. Griffith [6] was first to point out that Goodwin’s equations (1)-(3) admit oscillatory solutions only if , a very restrictive condition, because in experimental studies it is rare that more than 3 or 4 protein molecules bind cooperatively to DNA regulatory sequences [7]. This condition becomes even more restrictive if [4].

One solution: a longer feedback loop. The restriction *p* > 8 can be ameliorated by lengthening the feedback loop: if *n* = number of variables in the feedback loop, then the necessary condition for oscillations becomes . For example, for *n* = 8, the condition is *p* > 1.88. Longer loops (*n* > 3) correspond to inserting more than one intermediate (say, Y0, Y1, …, Y*n*−3) between X (mRNA) and Z (feedback component). This is quite reasonable, considering that PER protein has multiple phosphorylation sites [8]. Each intermediate, Y*j*, then denotes cytoplasmic PER phosphorylated on *j* sites, *j* = 0, 1, …, *J*. Eventually, the fully phosphorylated form, Y*J*, is transported into the nucleus and becomes Z. In this case, Goodwin’s dimensionless differential equations become Eqs. (4)-(7).

|  |  |  |
| --- | --- | --- |
|  |  | (4) |
|  |  |  |
|  |  | (5) |
|  |  |  |
|  |  | (6) |
|  |  |  |
|  |  | (7) |

Exactly the same equations can be derived by assuming a distributed time lag between *x* and *z* [9]

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

Introduce the new variables

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

For *j* = 0, we have Eq. (5) for *dy*0/*dt*. For *j* ≥ 1, *Gj*(0) = 0 and d*Gj*(*u*)/d*u* = *Gj*−1(*u*)− *Gj*(*u*); so we have Eq. (6) for *dy*j/*dt* for and Eq. (7) for *dz*/*dt*.

A second solution: Michaelis-Menten degradation of Z. In 1982 Bliss, Painter and Marr [10] proposed to replace the first-order degradation of Z, by a Michaelis-Menten rate law, where is the ‘Michaelis’ constant of the enzyme-catalyzed reaction and is the ‘*V*max’ of the reaction. With this change, the Goodwin model can exhibit limit cycle oscillations even for *p* = 1 [10]. The substitution of Michaelis-Menten rate laws for the first-order kinetic terms in Eqs. (1)-(3) has been exploited by many authors [11-13] to increase the robustness of their models of circadian rhythms.

# Kim & Forger’s extended models

In addition to the SNF model, Kim & Forger proposed two extended models, in which the core negative feedback loop involving PER and BMAL1 is supplemented with (either) an additional negative feedback from REV-ERB on transcription of the *Bmal1* gene (Figure 2b) (or) an additional positive feedback from ROR on transcription of the *Bmal1* gene (Figure 2c) [14]. Both extended models include the ODEs of the core SNF model.

Kim-Forger NNF Model. Equations (1)-(4) of the main text, plus

|  |  |  |
| --- | --- | --- |
|  |  | (10) |
|  |  | (11) |

where *V* is the (scaled) concentration of REV-ERB, *V*maxis the maximum achievable concentration of REV-ERB, *V*o is the REV-ERB concentration that would result in *A*T = 1 at steady state, and *δ* is a rate constant that sets the time scale for the feedback loop.

Kim-Forger PNF Model. Equations (1)-(4) of the main text, plus

|  |  |  |
| --- | --- | --- |
|  |  | (12) |
|  |  | (13) |

where *R* is the (scaled) concentration of ROR, and *R*max, *R*o and *δ* are defined similarly as in the NNF equations. For simulations of the NNF and PNF models, Kim & Forger chose *δ* = 0.2, *V*max = *R*max = 5, and they adjusted *V*o and *R*o to make the NNF and PNF models have the same average activator concentration, <*A*T>, as the SNF model.

# Non-dimensionalization of the modified Kim-Forger equations

The models presented in this paper, as well as Kim and Forger’s original models, were cast in non-dimensional form before simulation and analysis. For example, we show how to non-dimensionalize the SNF(1M8) model:

|  |  |
| --- | --- |
| **Dimensional Equations** | **Non-dimensional Equations** |
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|  |  |
|  |  |

where we have already set , because this constraint makes oscillations most likely. Species P1 … P6 represent both mRNA species (say, P1 = mature mRNA in nucleus, P2 = mRNA in cytoplasm) and PER proteins in the cytoplasm (say, P3 = unphosphorylated PER, P4 = monophosphorylated PER, etc.), and P = nuclear PER. The purpose of non-dimensionalization is to ‘scale away’ as many of the kinetic parameters as possible, to reduce the dimensionality of the space of independent parameters. To this end, we make the following change of variables from ‘hat-wearing’ variables (carrying physical units) to their respective dimensionless versions:

, , , …, , ,

The dimensionless ODEs (above right) are governed by five dimensionless parameters:

, , , , and .

# Estimation of a physically realistic value for

To determine a physically realistic value for the dissociation constant, , we must estimate the rate constants for binding and unbinding of the PER:BMAL complex. The upper limit for *k*bind is set by the Smoluchowski rate constant [15] for the diffusion-limited collision of two spherical molecules with diffusion coefficients *D*1and *D*2, and radii *R*1and *R*2:

, where (17)

and *N*A is Avogadro’s number. For reactant molecules with similar sizes and diffusion coefficients, Eq. (17) can be simplified to

|  |  |  |
| --- | --- | --- |
|  | ; | (18) |

hence, the Smoluchowski rate constant for the association of two proteins with radii 2 nm and diffusion constants of 15 μm2s−1 would be ~109 M−1s−1. Smoluchowski’s equation gives the maximum possible binding rate constant, when every collision between reactants leads to binding. For most molecules, especially proteins, the probability of reaction upon collision is much lower, because the proteins’ binding sites are likely limited to a fraction of the total surface area and successful binding also depends on details of molecular forces at atomic scales. The typical range of measured binding rates between proteins is 0.5‒5×106 M−1s−1 [16].

The rate constant, *k*unbind, for the dissociation of PER:BMAL complexes can be estimated from the residence time of the complex, which is likely to be on the order of minutes. So, 0.001 s−1 < *k*unbind < 0.01 s−1. With the previously derived diffusion-limited binding constant, *k*bind ≈ 1 nM−1s−1, the absolute lower limit on the dissociation constant is is in the range 10−3 to 10−2 nM. However, because *k*bind is typically at least three orders of magnitude lower than the Smoluchowski limit [16], a more realistic range for is 1 to 10 nM. ~~Recent measurements of the binding of PER:CRY to BMAL:CLOCK [17] suggest that is considerably larger than 10 nM, so we propose that   
10 nM < < 100 nM is a reasonable range for the value of this binding constant. Our estimated value 2 nM for the simulations in Figure S1 lies close to this range.~~

# Deriving the rate laws for *PER* transcription

BMAL:CLOCK binds to many E-box sequences throughout the mammalian genome, and PER:CRY binds to both free and E-box-bound BMAL:CLOCK complexes. Let E*i*, *i* = 1, …, Ω, denote all the E-box sequences that bind all four proteins BMAL, CLOCK, PER and CRY, where Ω ≈ 1500 according to ChIP-seq data [18]. The total concentrations of BMAL:CLOCK (A) and PER:CRY (P) dimers are:

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

|  |  |  |
| --- | --- | --- |
|  |  | (11) |

We are assuming that the synthesis and degradation of proteins are much slower reactions than the association and dissociations of proteins in a complex, so the total amounts of A and P in the system can be treated as constants on the time scale of the binding and unbinding reactions.

Because the total number of E-boxes (~1500) is considerably less than the total number of BMAL:CLOCK and PER:CRY complexes (~25,000 and ~30,000, respectively [19]), we can reasonably neglect the E-box-bound forms of the protein complexes, and assume that

|  |  |  |
| --- | --- | --- |
|  |  | (12) |

|  |  |  |
| --- | --- | --- |
|  |  | (13) |

To derive an expression for the rate of *PER* transcription, we need to estimate the fraction of E-boxes bound to BMAL:CLOCK but not to PER:CRY, i.e., [A:E*p*]/[E*p*]T, where *p* is the index corresponding to E-boxes driving *PER* gene expression. To this end, we consider the equilibrium binding reactions:

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

|  |  |  |
| --- | --- | --- |
|  |  | (15) |

|  |  |  |
| --- | --- | --- |
|  |  | (16) |

|  |  |  |
| --- | --- | --- |
|  |  | (17) |

The equilibrium dissociation constants of Reactions (14)-(17) are defined by

|  |  |  |
| --- | --- | --- |
|  |  | (18) |

The principle of detailed balance at equilibrium requires that

|  |  |  |
| --- | --- | --- |
|  |  | (19) |

That is, the dissociation constants for Reactions (14)-(17) satisfy

|  |  |  |
| --- | --- | --- |
|  |  | (20) |

Taking Eq. (20) into account, we are left with only three independent chemical equilibrium equations. One of these equations is

|  |  |  |
| --- | --- | --- |
|  |  | (21) |

which can be solved for the unknown concentration of A:P,

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

Meanwhile, the total number of *PER* E-boxes, [E*p*]T = [E*p*] + [A:E*p*] + [P:A:E*p*], can be written as:

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

which can be rearranged to give the probability that a *PER* gene is being transcribed:

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

Plugging the definition of *K*dAP1 (Eq. (18)) into Eq. (24) yields

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

First Case. PER:CRY binds equally strongly to free- and E-box-bound BMAL:CLOCK, i.e.,

|  |  |  |
| --- | --- | --- |
|  |  | (26) |

In this case, Eq. (25) becomes

|  |  |  |
| --- | --- | --- |
|  |  | (27) |

Plugging Eq. (12) into Eq. (27) yields Rate Law 1:

|  |  |  |
| --- | --- | --- |
|  |  | (28) |

Second Case. PER:CRY binds equally strongly to free- and E-box-bound BMAL:CLOCK, Eq. (26) (First Case above), and at the same time, BMAL:CLOCK saturates the *PER* E-box, i.e.,

|  |  |  |
| --- | --- | --- |
|  |  | (29) |

In this case, Eq. (28) becomes Rate Law 0 in the original Kim-Forger model:

|  |  |  |
| --- | --- | --- |
|  |  | (30) |

Third Case. BMAL:CLOCK cannot or can hardly bind PER:CRY and E-box simultaneously, i.e.,

|  |  |  |
| --- | --- | --- |
|  |  | (31) |

In this case, Eq. (25) gives rise to Rate Law 2:

|  |  |  |
| --- | --- | --- |
|  |  | (32) |

Electron microscopy studies by Aryal et al. have shown that PER:CRY::BMAL:CLOCK complexes bind to E-boxes [20], indicating that rate laws 0 and 1 are to be preferred to rate law 2.

**Optimization of SNF(1M8)**

Criteria:

1. Relative amp of Ptot > 0. 5 (for robust oscillation)
2. max(Ptot) is minimized (to minimize Kdhat)

Number of parameter sets: 1014

|  |  |  |
| --- | --- | --- |
| **SNF(1M8) optimization results.\*** | | |
| A | B | C |
| D | E | F |
| G | H | I |
| \*Red dots denote top-ten parameter sets (with smallest values of max(Ptot). | | |

Main findings:

1. Max(Ptot) = 30 ± 5 (panel A), Period = 21 ± 5 (panel B).
2. Period is strongly negatively correlated with beta\_max (panel C). This is not surprising, since smaller values of beta\_max mean a longer time delay for degrading nuclear PER and reinstituting *Per* gene transcription. The top 10 sets generate intermediate periods between 20 and 23.
3. Low max(Ptot) requires low Km and beta\_max (panels D, E, F). Lower values in both parameters slow down PER degradation, introduce time delay and increase robustness of oscillation (allowing oscillation to happen for larger values of Kd).
4. Alpha and AT are negatively correlated (panel G). This result is opposite to the trend in L models (Fig. 6b, although we are comparing different types of diagrams).
5. AT must be greater than ~KA/100 (panel H).
6. Alpha and KA positively correlated, and alpha must be greater than ~10sqrt(KA) (panel I), presumably to have sufficiently rapid transcription of *Per* gene as KA increases.

**Optimization of NNF(1M8)**

Criteria:

1. Relative amp of Ptot > 0.5
2. max(Ptot) is minimized
3. Relative amp of BMAL > 0.2
4. max(Rev) < 10
5. max(AT) / max(Ptot) as close to 1 as possible

Number of parameter sets: 1011

|  |  |  |
| --- | --- | --- |
| **NNF(1M8) optimization results.\*** | | |
| A | B | C |
| D | E | F |
| G | H | I |
| J | K | L |
|  |  |  |
| \*Red dots denote top-ten parameter sets (with smallest values of max(Ptot). | | |

Main findings:

1. Max(Ptot) = 80 ± 30 (panel A), Period = 23 ± 3 (bimodal, panel B).
2. Period is not strongly correlated with beta\_max (panel C), which is surprising.
3. Low max(Ptot) requires low Km and beta\_max (panels D, E, F), but not as strongly as in SNF.
4. Unlike SNF models, alpha and AT are no longer correlated (panel G)
5. With regard to the time-scale parameter, delta, there appear to be two clusters, one with delta <1 and another with delta >1 (panel H, I). The majority of top sets have delta < 1. In comparison to the delta < 1 cluster, the delta >1 cluster is associated with smaller values of VMAX and larger amplitudes of V(t) (panels J, K).
6. AMAX is positively correlated with VMAX (panel L).

**Optimization of PNF(1M8)**

Criteria:

1. Relative amp of Ptot > 0.5
2. max(Ptot) is minimized
3. Relative amp of BMAL > 0.2
4. max(ROR) < 5
5. max(AT) / max(Ptot) as close to 1 as possible

Number of parameter sets: 952

|  |  |  |
| --- | --- | --- |
| **PNF(1M8) optimization results.\*** | | |
| A | B | C |
| D | E | F |
| G | H | I |
| J | K | L |
|  |  |  |
| \*Red dots denote top-ten parameter sets (with smallest values of max(Ptot). | | |

Main findings:

1. Max(Ptot) = 4 ± 2 (panel A), Period = 35 ± 15 (panel B).
2. Period is strongly negatively correlated with beta\_max (panel C), as in SNF model.
3. Low max(Ptot) still requires low beta\_max, but is less dependent on Km (panels D, E, F).
4. Like the NNF model, alpha and AT are no longer correlated (panel G)
5. For most of the optimized parameter sets the time-scale parameter, delta, is > 1 (panel H), and in all cases eps << 1 (panel I), where eps is the ‘background’ rate of *Per* gene transcription when R(t) = 0.
6. AMAX and RMAX are strongly negatively correlated (panel J).
7. RMAX and alpha are positively correlated (panel K), and alpha and KA are strongly positively correlated (panel L).

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