A Coupled Stochastic Model Explains Differences in Circadian Behavior of Cry1 and Cry2 Knockouts

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November 18, 2014

Classification:
Quantitative Biology | Molecular Networks

Keywords:
Systems Biology | Circadian Rhythms | Stochastic Systems
1 Abstract

In the mammalian suprachiasmatic nucleus (SCN), a population of noisy cell-autonomous oscillators synchronizes to generate robust circadian rhythms at the organism-level. Within these cells two isoforms of Cryptochrome, Cry1 and Cry2, participate in a negative feedback loop driving circadian rhythmicity. Previous work has shown that single, dissociated SCN neurons respond differently to Cry1 and Cry2 knockouts: Cry1 knockouts are arrhythmic while Cry2 knockouts display more regular rhythms. These differences have led to speculation that CRY1 and CRY2 may play different functional roles in the oscillator. To address this proposition, we have developed a new coupled, stochastic model focused on the Period (Per) and Cry feedback loop, and incorporating intercellular coupling via vasoactive intestinal peptide (VIP). Due to the stochastic nature of molecular oscillations, we demonstrate that single-cell Cry1 knockout oscillations display partially rhythmic behavior, and cannot be classified as simply rhythmic or arrhythmic. Our model demonstrates that intrinsic molecular noise and differences in relative abundance, rather than differing functions, are sufficient to explain the range of rhythmicity encountered in Cry knockouts in the SCN. Our results further highlight the essential role of stochastic behavior in understanding and accurately modeling the circadian network and its response to perturbation.

2 Introduction

Circadian rhythmicity in gene expression affects a wide variety of biological processes at multiple scales, including sleep-wake cycles, body temperature, feeding-fasting behavior, cell cycles, and metabolism. A population of approximately 20,000 oscillators in the suprachiasmatic nucleus (SCN) of the brain serves as the mammalian master clock, and synchronizes circadian oscillations in peripheral tissues. Cellular oscillators in the SCN display stochastic variation in rhythmicity and period length, due to the low molecular counts of core clock proteins. Stochastic noise has previously been implicated in a variety of phenomena within the SCN, and is thought to play an important role in SCN behavior. SCN cells resist noisy behavior and establish robust system-wide rhythmicity through intercellular coupling via the neurotransmitter vasoactive intestinal peptide (VIP) that promotes expression of the circadian gene Period (Per).

The mammalian cell-autonomous oscillator is driven by a central transcription-translation feedback loop, in which the protein products of E box-activated genes Per and Cryptochrome (Cry) form heterodimers to repress their own transcription, as shown in Fig. 1. As these proteins are degraded, transcription is reactivated, repeating the cycle with near-24 hour periodicity. While both CRY1 and CRY2 isomers are known E box repressors, Cry1 and Cry2 knockouts display significant differences in rhythmicity and period length. Prior work has shown that single dissociated SCN neurons display persistent circadian rhythmicity in wild-type and Cry2 knockout, while dissociated Cry1 knockout cells are largely arrhythmic. However, rhythmicity may be restored in Cry1 knockout populations through intercellular coupling. This difference in knockout behaviors has led to speculation that CRY1 and CRY2 play distinct and non-redundant roles in the circadian oscillator. Other reasons for this effect have also been
Figure 1: Visualization of model components. Our model explicitly includes four mRNA species (Per, Cry1, Cry2, VIP), four cytosolic proteins (PER, CRY1, CRY2, VIP), and three nuclear transcription regulators (PER-CRY1, PER-CRY2, and CREB). The central negative feedback loop (via PER-CRY) is modulated by the external positive feedback loop (via VIP) which mediates cell to cell communication. VIP excreted from nearby cells is received by G protein-coupled receptor (GPCR), which couples cells in proximity to one another by diffusion.

proposed, including differing strength of repression, and phase delays [16,17].

Here, we address the roles of Cry1 and Cry2 through a coupled stochastic model of the core circadian oscillator, explicitly accounting for intrinsic noise and coupling dynamics through the VIP and C-response element binding protein (CREB) pathway. Our model accurately captures both dissociated cell and population-level behavior of the circadian network. We demonstrate that it is not necessary for CRY1 and CRY2 to perform different functions in the oscillator; rather, knockout characteristics of SCN neurons are well-explained by the stoichiometry of PER, CRY1, and CRY2 proteins, in conjunction with intrinsic noise. Specifically, the higher expression of Cry1 allows dissociated Cry2 knockout cells to retain noise-induced rhythmic behavior, whereas dissociated Cry1 knockouts do not have sufficient CRY concentrations to rhythmically repress transcription. When coupled, the positive-feedback VIP loop forces cells to cross a bifurcation and become limit cycle oscillators, as positive feedback loops under certain conditions promote oscillatory behavior [18]. Our results reconcile single-cell and network behavior, and demonstrate the importance of stoichiometry and stochastic noise in understanding the circadian oscillator.

3 Model Structure and Parameter Identification

A schematic representation of the states captured in our model is shown as Fig. 1. We focus on accurately capturing the network dynamics of the central Per-Cry transcription-translation feedback loop. For intercellular coupling, we include the VIP-CREB pathway, as VIP has been shown to play an essential role in driving synchronization within the SCN through induction of the core clock gene Per [9-11].

Our model explicitly includes the dynamics of the PER-CRY1-CRY2 feedback loop and VIP
signalling, resulting in an 11-state model with 34 kinetic parameters. The differential equations and the corresponding stochastic propensity functions for each state were formulated using Michaelis-Menten kinetics for repression and enzyme-mediated degradation, and mass-action kinetics for translation and dimerization reactions. For Per, an additional CREB-dependent activation term was added to the Michaelis-Menten repression. Differences between Cry1 and Cry2 expression levels are captured through differing transcription, translation, and degradation rates. Despite some evidence suggesting a difference in repressive ability between CRY isoforms (19), our model did not require explicitly differentiating between repressive potency to accurately capture experimental phenotypes.

We optimized model parameters using an evolutionary algorithm. The optimization was performed with respect to experimental mRNA and protein stoichiometry, amplitudes, and phase relationships (13, 20–23). Additional data from RNAi experiments (24) were also used to ensure correct period sensitivity. To reduce computational cost, parameters were estimated based on the deterministic model. Since a majority of quantitative biological data exist at population level, we optimized the model in the coupled state with the VIP pathway included. To model uncoupled cells, all VIP concentrations were set to zero, which reflects the low concentration of VIP and lack of functional synapses in cultures of mechanically-dissociated SCN neurons. For conversion to a stochastic model, the volume parameter $\Omega$ was fit to the desynchronization rate of decoupled oscillators to capture the correct amount of intrinsic stochastic noise (25).

The model equations for each state were written and solved using the CasADi computer algebra package (26) and the SUNDIALS ODE solvers suite (27). The model was simulated stochastically using the Gillespie algorithm, as implemented in StochKit2 (28). A full description of model equations and parameters is included in the Appendix.

4 Results

In the single-cell case, the deterministic model shows damped oscillations (Fig. 2(a)). As shown in (30), single dissociated neurons are well approximated as damped oscillators driven by molecular noise, and cannot be distinguished from noisy limit-cycle oscillators. Indeed, when simulated stochastically (Fig. 2(c)), single dissociated cells display sustained non-damping oscillatory behavior. With the VIP coupling pathway included, the system crosses a bifurcation to a deterministic limit cycle oscillator (Fig. 2(b)). When simulated stochastically in Fig. 2(d), this coupling is sufficient to resist stochastic noise at a population level and drive phase synchrony and stable oscillation in the mean (black), despite stochastic noise in the single-cell trajectories (gray traces).

The central clock in the SCN has shown a strong robustness to genetic perturbations, and the ability to maintain synchronized rhythmicity even with knockouts to core clock genes (8, 12). At a single-cell level the clock is less robust to perturbation, with dissociated Cry1 knockouts displaying less regular rhythms. First we demonstrate that our model captures the phenotypes of Cry1 and Cry2 knockouts at both the level of dissociated cells (Fig. 3) and coupled networks (Fig. 4). If both CRY isoforms act as E-box repressors, knockout of either CRY is expected to increase transcription of Per, with the response being more severe for the Cry1 knockout, as CRY1 is more abundant than CRY2. This is indeed seen in both Per2-luc measurements (12) and our in silico Per mRNA trajectories. When coupling is restored as in full SCN explants, rhythmicity and synchronization is restored for Cry1 knockouts in both experiment and simulation, as shown in Fig. 4. The model also correctly captures the directions of population-level period sensitivity to Cry knockouts. Model Cry1 knockouts displayed a shortened period 81% of wild-type length, while model Cry2 knockouts displayed a lengthened period 114% of wild type length, consistent
Figure 2: Deterministic ((a)-(b)) and stochastic ((c)-(d)) simulation of the single-cell and network-level model. Deterministic trajectories represent normalized Per mRNA concentrations, stochastic trajectories show Per mRNA molecule count. (a) As in (29, 30), the deterministic model shows damped oscillations. (b) When coupling (VIP) is included, the model crosses a bifurcation to a deterministic limit-cycle oscillator. (c) Despite showing damped oscillations in the deterministic model, stochastic simulation of the single-cell model demonstrates no decay in noise-driven oscillation amplitude. (d) Despite intrinsic molecular noise, synchronization is maintained at a mean population level (n = 225 cells, black). Individual cell traces are shown in gray.

Figure 3: Our stochastic mathematical model captures individual neuron behavior. (a)-(c) Experimental Per2-luc traces, from (12). (d)-(f) Per mRNA stochastic simulation trajectories from our model. In (12), the authors show that Cry1 and Cry2 knockout cells display increased bioluminescence compared to wild type. This result is in agreement with our model predictions (309% of WT for Cry1 knockout, 104% of WT for Cry2 knockout), as a higher level of PER is required to repress transcription in order compensate for lower CRY levels.
Figure 4: Rhythmicity and synchronization were achieved for coupled SCN phenotypes in both experimental SCN explants (12) ((a)-(c)) and in silico coupled simulations ((d)-(f)). Individual cell traces for simulation data are shown in gray. Both sets of trajectories are normalized, as the number of cells differs between experimental SCN explants. Period shortening (Cry1 KO) and lengthening (Cry2 KO) are observed in simulation results, capturing experimental knockout behavior. A downward trend is observed in biological SCN explant rhythms due to cell death.

with (12,13,31).

To quantify neuron behavior, we use a rhythmicity index as defined in (32): the fraction of energy contained within the frequency band corresponding to circadian oscillations, as calculated via a discrete wavelet transform (DWT). The discrete wavelet transform is performed by applying high and low-pass filters consecutively to resolve a time series at different scales. The resulting set of wavelet coefficients can be used to reconstruct the original signal at each frequency scale. The energy $\|D_j\|^2$ for each DWT level $j$ is

$$\|D_j\|^2 = \sum_{k=1}^{K} W_{j,k}^2,$$  

where $W_{j,k}$ is the $k^{th}$ wavelet coefficient at DWT level $j$. Thus, the rhythmicity index is

$$RI = \frac{\|D_c\|^2}{\sum_{j=1}^{J} \|D_j\|^2},$$  

where $J$ is the number of levels of the discrete wavelet transform, and $D_c$ is the energy of the wavelet level containing the circadian frequency. By Parseval’s theorem, this is equivalent to the mean of the squared Fourier coefficients within each frequency band (32). We define our circadian frequency band $D_c$ to contain periods between $\frac{2}{3}$ and $\frac{4}{3}$ of mean wild-type period, as it will correctly capture stochastic oscillations with a near-circadian frequency.

When this metric is applied (Fig. 5), experimental and simulated results show good agreement, and demonstrate the strong increase in circadian rhythmicity in coupled Cry1 knockouts.
Figure 5: Model correctly predicts noise characteristics of single-cell and coupled network SCN neurons. Black indicates single-cell uncoupled rhythmicity index, gray indicates coupled population-level rhythmicity, as defined in Equations 1 and 2. Error bars represent variance. (a) Stochastic simulation results. Single cell mean and variance were taken from 225 cells of each phenotype. Coupled population rhythmicity was taken from a population of 225 cells, sufficiently large to approximate SCN tissue. Single-cell Cry1 knockouts display less rhythmicity in the circadian frequency band, which is restored at a population level through coupling. (b) Experimental results from [12]. Single-cell mean and variance were taken from ten cells of each phenotype. SCN tissue rhythmicity was taken from tissue explants of $O(10^3)$ cells. Overall knockout behavior corresponds closely with model prediction.

Since Cry1 and Cry2 play the same role in our model, our result indicates that the relative abundance of Cry isoforms in conjunction with intrinsic molecular noise is sufficient to capture the differing effects of Cry1 and Cry2 knockouts.

5 Conclusion

In this work, we applied a new coupled stochastic model of the circadian oscillator to a lingering question regarding the roles of Cry isoforms. We demonstrate that relative abundance is sufficient to explain Cry knockout behaviors, and that a parallel role for Cry1 and Cry2 in the circadian gene repression is consistent with experimental data. In this work, intercellular coupling is shown to improve rhythmicity by means of synchronization. Our results suggest that this coupling shifts cells from noise-driven oscillators to stochastic limit cycles, thus improving resistance to perturbation. This further supports the possibility that single, decoupled cells may not exhibit deterministic limit cycles, though single-cell limit cycle oscillators remain a widely-used convention. Finally, our results emphasize the importance of describing circadian rhythmicity at the single-cell level with a continuous metric, rather than the traditional binary classification of “rhythmic” or “arrhythmic.” Though our chosen rhythmicity metric is not new, it adds additional detail to our understanding of cellular circadian behavior, and further validates the dynamics captured by this new model.
6 Appendix: Model Description

Deterministic Model

Our deterministic model is composed of eleven ODEs. Each reaction in the model obeys either mass-action or Michaelis-Menten kinetics, and the system behaves as a limit cycle oscillator without requiring higher-order Hill kinetics. This reflects (18), in that a positive feedback loop (VIP-CREB promotion of Per transcription) ameliorates the need for physically unrealistic high order Hill terms. Avoiding Hill terms decreases error in the stochastic simulation, as the linear noise approximation (LNA) variance of the stochastic Michaelis-Menten equation is known to be bounded to $[1, \frac{4}{3}]$ of the true LNA variance at steady state (33).

The period of the deterministic model was scaled to 23.7 hours, however, the amplitudes were not scaled. The units of the state variables are arbitrary relative concentrations, and the units of parameters are provided. Tables 1 and 2 contain the equations and parameters, respectively, for our ODE model of circadian cells.

Model Assumptions

The following assumptions were made in the development of this model:

- CLOCK and BMAL1 are considered approximately constant, through the $v_{2pr}$ parameter. This parameter captures the repression of E-box genes, which is achieved through binding of PER-CRYs to CLOCK-BMAL1.

- Degradation within the cell is assumed to be enzyme-mediated with Michaelis-Menten kinetics. Extracellular VIP is assumed to degrade with mass-action kinetics.

- No Hill term of order higher than unity is used, as the external positive feedback arm causes sufficient nonlinearity to create a limit cycle.

- Because nuclear CRY1 and CRY2 are believed to share a degradation pathway, shared degradation kinetics are derived under the pseudo-steady state hypothesis, as in (34).

- PER is assumed to be required for nuclear uptake of CRY. We additionally assume that equilibrium between PER-CRY complexes and free CRY proteins in the nucleus is fast, and do not explicitly consider multiple CRY nuclear states.

Conversion to Coupled Stochastic Form

To create a stochastic form of the model, concentration was converted to population using a volume parameter $\Omega$. This volume was determined by fitting our stochastic model to the desynchronization rate of a population of decoupled oscillators, to capture the correct amount of stochasticity in the population. For our model, $\Omega = 400$. Additionally, a diffusion rate between adjacent cells was defined, and an equation added to account for coupling between cells. Networks for population simulations consisted of 225 cells. Coupled populations were placed on a 15-by-15 cell grid, and coupled to the two nearest neighbors horizontally and vertically. Boundary conditions were considered to be periodic. VIP in the grid location of one cell ($VIP_1$) may move to a coupled cell ($VIP_2$) with propensity $k_t \times VIP_1 \Omega$, in units molecules/hour. We used a $k_t$ value of 10.0 to reflect the fact that diffusion occurs rapidly in comparison to the slow reactions involved in cellular timekeeping. However, $k_t$ values within an order of magnitude yield near-identical results.
Model Fitting

Our model was fit by use of an evolutionary algorithm in Python (35). Parameter values were generated randomly. Criteria used in the fitness function constructed for this algorithm are shown in Table 3. If the solution for a set of parameter values was not a limit-cycle oscillator, the fitness function returned a maximum value.
Table 1: Ordinary differential equations comprising the coupled circadian model.

| State Variable | Symbol | Model Equation |
|----------------|--------|----------------|
| Per mRNA       | p      | \( \frac{dp}{dt} = \frac{v_{1p}^{\text{CREB}} + v_{2pr}}{K_{1p} + C1P + C2P} - \frac{v_{3p}p}{K_{2dP} + p} \) |
| Cry1 mRNA      | c1     | \( \frac{dc1}{dt} = \frac{v_{4c1r}}{K_{3c} + C1P + C2P} - \frac{v_{5c1}c1}{K_{4dc} + c1} \) |
| Cry2 mRNA      | c2     | \( \frac{dc2}{dt} = \frac{v_{6c2r}}{K_{3c} + C1P + C2P} - \frac{v_{7c2}c2}{K_{4dc} + c2} \) |
| VIP mRNA       | vip    | \( \frac{dvip}{dt} = \frac{v_{8sr}}{K_{5v} + C1P + C2P} - \frac{v_{9v}vip}{K_{6dv} + vip} \) |
| Per Protein    | P      | \( \frac{dP}{dt} = k_{1p}P - \frac{v_{10P}P}{K_{8dp} + P} - v_{11aCP}P \times C1 - v_{11aCP}P \times C2 \) + \( v_{12dCP}C1P + v_{12dCP}C2P \) |
| Cry1 Protein   | C1     | \( \frac{dC1}{dt} = k_{2c}c1 - \frac{v_{13C1}C1}{K_{9dc} + C1} - v_{11aCP}P \times C1 + v_{12dCP}C1P \) |
| Cry2 Protein   | C2     | \( \frac{dC2}{dt} = k_{2c}c2 - \frac{v_{14C2}C2}{K_{9dc} + C2} - v_{11aCP}P \times C2 + v_{12dCP}C2P \) |
| VIP Protein    | VIP    | \( \frac{dVIP}{dt} = k_{3v}vip - v_{15v}VIP \) |
| CRY1-PER Dimer | C1P    | \( \frac{dC1P}{dt} = v_{11aCP}P \times C1 - v_{12dCP}C1P - \frac{v_{16C1P}C1P}{K_{10dCn} + C1P + C2P} \) |
| CRY2-PER Dimer | C2P    | \( \frac{dC2P}{dt} = v_{11aCP}P \times C2 - v_{12dCP}C2P - \frac{v_{17C2P}C2P}{K_{10dCn} + C1P + C2P} \) |
| CREB Protein   | CREB   | \( \frac{dCREB}{dt} = \frac{v_{18v}VIP}{K_{11V} + VIP} - \frac{v_{19CR}CREB}{K_{12dCR} + CREB} \) |
Table 2: Parameter descriptions for circadian ODE model.

| Parameter | Description                                      | Value  | Units       |
|-----------|---------------------------------------------------|--------|-------------|
| $v_{1pp}$ | CREB-induced Per mRNA promotion                   | 0.235  | [-]/hr      |
| $v_{2pr}$ | Per mRNA transcription                            | 0.415  | [-]/hr      |
| $v_{3p}$  | Per mRNA degradation                              | 0.478  | [-]/hr      |
| $v_{4c1r}$| Cry1 mRNA transcription                           | 0.350  | [-]/hr      |
| $v_{5c1}$ | Cry1 mRNA degradation                             | 1.44   | [-]/hr      |
| $v_{6c2r}$| Cry2 mRNA transcription                           | 0.124  | [-]/hr      |
| $v_{7c2}$ | Cry2 mRNA degradation                             | 2.28   | [-]/hr      |
| $v_{8vs}$ | VIP mRNA transcription                             | 0.291  | [-]/hr      |
| $v_{9v}$  | VIP mRNA degradation                              | 1.35   | [-]/hr      |
| $v_{10p}$ | Per protein degradation                           | 13.0   | [-]/hr      |
| $v_{11aCP}$| PER-CRY dimer formation                           | 0.493  | ([-]× hr)$^{-1}$ |
| $v_{12dcP}$| PER-CRY dimer dissociation                        | 0.0080 | 1/hr        |
| $v_{13c1}$| Cry1 protein degradation                          | 4.12   | [-]/hr      |
| $v_{14c2}$| Cry2 protein degradation                          | 0.0862 | [-]/hr      |
| $v_{15V}$ | VIP protein degradation                           | 0.723  | 1/hr        |
| $v_{16c1P}$| PER-CRY1 dimer degradation                        | 0.0306 | [-]/hr      |
| $v_{17c2P}$| PER-CRY2 dimer degradation                        | 0.840  | [-]/hr      |
| $v_{18V}$ | CREB activation by VIP receptors                  | 0.789  | [-]/hr      |
| $v_{19CR}$| CREB deactivation                                 | 1.27   | [-]/hr      |
| $k_{1p}$  | PER translation                                   | 7.51   | 1/hr        |
| $k_{2c}$  | CRY translation                                    | 0.572  | 1/hr        |
| $k_{3v}$  | VIP translation                                    | 5.50   | 1/hr        |
| $K_{1p}$  | Per transcription constant                         | 0.264  | [-]         |
| $K_{2dp}$ | Per degradation constant                           | 0.00795| [-]         |
| $K_{3c}$  | Cry transcription constant                         | 0.156  | [-]         |
| $K_{4dc}$ | Cry degradation constant                           | 1.94   | [-]         |
| $K_{5v}$  | VIP transcription constant                         | 0.115  | [-]         |
| $K_{6dv}$ | VIP degradation constant                           | 0.110  | [-]         |
| $K_{7dp}$ | Per protein degradation constant                   | 0.0372 | [-]         |
| $K_{8dc}$ | Cry protein degradation constant                   | 4.23   | [-]         |
| $K_{9dcP}$| PER-CRY dimer degradation constant                 | 0.0455 | [-]         |
| $K_{10V}$ | CREB protein activation constant                   | 1.46   | [-]         |
| $K_{11CR}$| CREB protein deactivation constant                 | 1.01   | [-]         |
Table 3: Components of the model fitness function for optimizing a parameter set. Criteria 1-11, 14-18 from \((31)\); criteria 12, 13 from \((12, 13, 31)\); criterion 20 from \((36)\); and criteria 19, 21 from \((37)\).

| Index | Description                              | Weight | Desired | Result |
|-------|------------------------------------------|--------|---------|--------|
| 1     | *Per* mRNA Peak-trough ratio             | 0.5    | Large   | Large  |
| 2     | *Cry1* mRNA Peak-trough ratio            | 0.5    | 2.16    | 2.30   |
| 3     | *Cry2* mRNA Peak-trough ratio            | 0.5    | 2.24    | 2.20   |
| 4     | *Per* protein Peak-trough ratio          | 3      | Large   | Large  |
| 5     | *Cry1* protein Peak-trough ratio         | 3      | 3.247   | 2.41   |
| 6     | *Cry2* protein Peak-trough ratio         | 3      | 1.98    | 1.60   |
| 7     | Fraction *PER* of total *PER, CRY1, CRY2*| 3      | 0.10    | 0.06   |
| 8     | Fraction *CRY1* of total *PER, CRY1, CRY2*| 3     | 0.56    | 0.63   |
| 9     | Fraction *CRY2* of total *PER, CRY1, CRY2*| 3   | 0.34    | 0.31   |
| 10    | *Cry1* siRNA sensitivity                 | 5      | < 0     | < 0    |
| 11    | *Cry2* siRNA sensitivity                 | 5      | > 0     | > 0    |
| 12    | *Cry1* knockout period                   | 12     | < 0.95  | 0.89   |
| 13    | *Cry2* knockout period                   | 12     | > 1.15  | 1.16   |
| 14    | Fraction *PER*-*CRY1* of total *CRY1*    | 1      | 0.40    | 0.22   |
| 15    | Fraction *PER*-*CRY2* of total *CRY2*    | 1      | 0.35    | 0.10   |
| 16    | $t_{\text{max}}$ mRNAs - $t_{\text{max}}$ complexes | 3    | 0.75    | 0.81   |
| 17    | $t_{\text{max}}$ cytosolic protein - $t_{\text{max}}$ mRNAs | 3 | 0.25    | 0.01   |
| 18    | $t_{\text{max}}$ PER-CRY - $t_{\text{max}}$ cytosolic protein | 3 | 0     | 0.18   |
| 19    | $t_{\text{max}}$ CREB - $t_{\text{max}}$ mRNAs | 8 | > 0.80 | 0.93   |
| 20    | *VIP* protein peak-trough ratio          | 5      | 3.00    | 3.48   |
| 21    | CREB peak-trough ratio                   | 5      | 3.00    | 2.76   |
| 22    | Deterministic coupled cells must synchronize | 20 | True  | True   |
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