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Emergence of Noise-Induced Oscillations in the Central Circadian Pacemaker

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Abstract

\textit{Bmal1} is an essential transcriptional activator within the mammalian circadian clock. We report here that the suprachiasmatic nucleus (SCN) of \textit{Bmal1}-null mutant mice, unexpectedly, generates stochastic oscillations with periods that overlap the circadian range. Dissociated SCN neurons expressed fluctuating levels of PER2 detected by bioluminescence imaging but could not generate circadian oscillations intrinsically. Inhibition of intercellular communication or cyclic-AMP signaling in SCN slices, which provide a positive feed-forward signal to drive the intracellular negative feedback loop, abolished the stochastic oscillations. Propagation of this feed-forward signal between SCN neurons then promotes quasi-circadian oscillations that arise as an emergent property of the SCN network. Experimental analysis and mathematical modeling argue that both intercellular coupling and molecular noise are required for the stochastic rhythms, providing a novel biological example of noise-induced oscillations. The emergence of stochastic circadian oscillations from the SCN network in the absence of cell-autonomous circadian oscillatory function highlights a previously unrecognized level of circadian organization.

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Abbreviations: CA, coupling agent; CAMP, adenosine 3’,5’-monophosphate; CRE, CAMP response element; CREB, CRE-binding protein; FFT, Fast Fourier transform; LUC, luciferase; PER2, Period2; SCN, suprachiasmatic nucleus; TTX, tetrodotoxin; VIP, vasoactive intestinal polypeptide; WT, wild type.

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Introduction

Mammalian circadian clocks are cell autonomous and self-sustained within the central pacemaker, the suprachiasmatic nucleus (SCN), as well as within peripheral tissues and fibroblasts [1–4]. Circadian rhythms are generated at the molecular level by an autoregulatory transcriptional and translational feedback loop [5,6]. The bHLH-PAS proteins, CLOCK (\textit{Clock} RefSeq: NM_007715) and BMAL1 (\textit{Bmal1} RefSeq: NM_007489), form an activator complex that drives transcription of the \textit{Per} (RefSeq: \textit{Per1} NM_011065 and \textit{Per2} NM_011066) and \textit{Cry} (RefSeq: \textit{Cry1} NM_007771 and \textit{Cry2} NM_009963) genes [7–9]. PER and CRY proteins then form repressor complexes and translocate back to the nucleus to inhibit their own transcription [10,11].

Previous work has demonstrated that \textit{Bmal1} plays an essential role in normal circadian clock function—its inactivation leads to a loss of circadian rhythmicity at the behavioral level [7]. To understand the role of \textit{Bmal1} at a more mechanistic level, we have analyzed the effects of a \textit{Bmal1} loss-of-function mutation (\textit{Bmal1} \textsuperscript{-/-}) on cell- and tissue-autonomous circadian rhythms in the SCN and peripheral tissues. We report here an unexpected stochastic rhythmicity in the SCN of \textit{Bmal1}-deficient mice, which appears as an emergent network property of the SCN.

The roles of intercellular coupling and molecular noise in circadian clock function have been analyzed using mathematical models [12–15]. Previous work on modeling the role of coupling
Author Summary

The suprachiasmatic nucleus (SCN) is the master circadian pacemaker in mammals that controls and coordinates physiological processes in a daily manner. The SCN is composed of a network of cells, with each cell acting as an autonomous oscillator. In isolated individual cells, time-keeping is not precise because of the inherent randomness in the biochemical reactions within each cell, involving its core clock components. However, in the SCN network, precise rhythms can emerge because of intercellular coupling. In this article, we study a loss-of-function mutation of BMAL1, a core clock component, which eliminates timekeeping in isolated cells. Surprisingly, in both experiments and mathematical simulations, we find that noisy rhythms emerge from the SCN network even in the presence of this BMAL1 mutation. This random yet coordinated timekeeping has not been observed in previous modeling and experimental work and indicates that a network of cells can utilize noise to help compensate for loss of a physiological function. In normal function, the SCN network mitigates any variability observed in individual cellular rhythms and produces a precise and rhythmic network timekeeping signal. When the individual cells are no longer rhythmic, the coupling pathways within the SCN network can propagate stochastic rhythms that are a reflection of both feed-forward coupling mechanisms and intracellular noise. Thus, in a manner analogous to central pattern generators in neural circuits, rhythmicity can arise as an emergent property of the network in the absence of component pacemaker or oscillator cells.

Results

Rhythmic PER2::LUC Output in Bmal1<sup>-/-</sup> SCN Explants

*Bmal1* is the only known clock gene whose loss-of-function leads to complete loss of circadian rhythmicity in wheel-running behavior [7]. In addition, circadian rhythms of *Per1* and *Per2* mRNA expression are disrupted in the SCN of *Bmal1*<sup>-/-</sup> mice [7]. To analyze the role of Bmal1 in cell- and tissue-autonomous circadian properties in central and peripheral oscillators, we measured PER2::LUC bioluminescence rhythms in various tissue explants from *Bmal1<sup>-/-</sup>* and homozygous *Bmal1* mutant (*Bmal1<sup>-/-</sup>*) mice. Surprisingly, a persistent, yet highly variable, rhythmicity emerged in *Bmal1<sup>-/-</sup>* SCN explants (Figure 1 and Video S1), whereas all peripheral tissues failed to generate PER2::LUC circadian rhythms (Figure 1A).

WT and *Bmal1<sup>-/-</sup>* SCN explants show persistent PER2::LUC bioluminescence rhythms for more than 35 d in culture (Figure 1B and 1C; also see Datasets S1 and S2). Fast Fourier transform (FFT) spectrograms of the records show a consistent frequency (about one cycle per day) for the WT SCN; however, more variable frequency components are observed in the *Bmal1<sup>-/-</sup>* SCN (Figure 1B and 1C, middle). Double-plotted raster plots illustrate the stable PER2::LUC rhythmicity in the WT SCN and the unstable rhythms in *Bmal1<sup>-/-</sup>* SCN (Figure 1B and 1C, right). FFT analysis shows that the dominant periodicity in WT SCN is tightly clustered around 24 h, while periodicity is much more variable, ranging from about 14 to 30 h, in *Bmal1<sup>-/-</sup>* SCN (Figure 1D). Because of the highly variable periods and instability of the rhythmicity from *Bmal1<sup>-/-</sup>* SCN, we refer to these fluctuations as stochastic. All tissues from WT littersmates, including the SCN, pituitary, liver, lung, and cornea, displayed normal circadian rhythms similar to those observed in previous studies (Figure 1A) [4,28].

Due to the stochastic nature of the rhythms in the *Bmal1<sup>-/-</sup>* SCN, period estimates based on averages of long time series do not adequately describe the cycle-to-cycle variability. Hence, we measured individual peak-to-peak intervals of the PER2::LUC expression patterns from each SCN explant (Figure 1E, left). The mean inter-peak intervals observed in the WT SCN explants were circadian at about 24 h (24.3±0.3 SD h). These values were similar to the periods estimated by Levenberg-Marquardt (LM) curve fitting of the entire time series using the Lumicycle Analysis Program (WT = 24.04±0.09 SD h, n = 23). By contrast, the *Bmal1<sup>-/-</sup>* SCN showed a significantly shorter average inter-peak interval length of 17.8 h with a much greater variance (SD = 5.54 h, n = 19) than WT SCN explants.

Using the inter-peak interval data, we determined the Serial Correlation Coefficient of successive intervals, r<sub>s</sub> (Figure 1E, right). A negative serial correlation reflects the likelihood that a long cycle will be followed by a short cycle, or vice versa, which is a characteristic feature of a functional pacemaker-driven system [39]. The average serial correlation coefficients for WT SCN explants were negative (mean r<sub>s</sub> = −0.17±0.06 SD h, t-test, p<0.01) as would be expected from a circadian pacemaker-driven process (Figure 1E, right). However, the coefficient for the *Bmal1<sup>-/-</sup>* SCN was slightly positive and marginally significant (mean r<sub>s</sub> = 0.07±0.03 SD h, t-test, p<0.05). This weak serial correlation coefficient differs from the pacemaker-driven processes seen in WT SCN explants and is consistent with either an oscillator with a highly labile period or a “random walk” process [40].

Isolated Bmal1<sup>-/-</sup> SCN Neurons Fail to Generate Circadian Rhythms

To determine whether the stochastic PER2::LUC rhythms from *Bmal1<sup>-/-</sup>* SCN explants are cell autonomous, we studied PER2::LUC bioluminescence at the single-cell level (Figure 2). We first imaged the overt bioluminescence expression patterns from *Bmal1<sup>-/-</sup>* SCN explants (Figure 2A, Video S1, Datasets S3 and S4) and analyzed bioluminescence from individual neurons...
Figure 1. Effects of Bmal1 mutation on PER2::LUC bioluminescence rhythms. (A) Representative records of PER2::LUC bioluminescence from various tissue explants from wild type (WT) and homozygous Bmal1-null mutant (−/−) mice. Mice were kept in a light-dark cycle (12 h light, 12 h dark) for approximately 2 wk, then released into constant darkness. The tissue explants were dissected (day 0) and immediately cultured for recording.
Data are shown following a medium change (day 8); another medium change occurred at day 15. (B and C) Detailed view of PER2::LUC bioluminescence from WT (B) and Bmal1−/− mice (C). Records begin on the day of culture (day 0) and are “raw” Lumicycle bioluminescence recordings that are not normalized nor corrected for baseline drift. SCN explants show persistent PER2::LUC rhythms for >35 d (left). FFT spectrograms of the baseline-subtracted records (middle) show a tightly regulated frequency (cycles per day) for the WT SCN; however, more variable frequency components are observed in the Bmal1−/− SCN. Double-plotted raster plots (right) illustrate stable PER2::LUC rhythms in the WT SCN and instability of the rhythms in Bmal1−/− SCN. (D) FFT spectral analysis for PER2::LUC rhythms from SCN explants. Period values with the maximum spectral power were determined for WT and Bmal1−/− SCN explants using FFT spectral analysis (see Methods under Single-Cell Imaging Data Analysis). Each data point represents the maximum frequency component in a 10-d epoch of data. Bmal1−/− SCN explants showed high variability in period length (average = 20.51 h) compared to WT SCN explants (average period = 24.16 ± 0.83 SD h). (D) Histograms of inter-peak intervals for the PER2::LUC rhythmic expression patterns (left) and serial correlation coefficient (right) of successive inter-peak intervals. The Bmal1−/− SCN explants show a significantly shorter average inter-peak interval and a much broader distribution compared to WT SCNS. Histograms represent 433 inter-peak intervals from 23 WT SCN explants and 1,239 intervals from 19 Bmal1−/− SCN explants. r_s estimates were calculated from successive 7 to 10 inter-peak interval epochs. Histograms represent 36 r_s estimates from 14 WT SCN explants and 85 r_s estimates from 16 Bmal1−/− SCN explants. The average serial correlation coefficient for WT SCN explants was negative (r_s = −0.17, p < 0.01) as would be expected from a circadian pacemaker-driven process. However, the coefficient for the Bmal1−/− SCN was slightly positive (r_s = 0.07, p < 0.05).

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(figure 2B and 2C). The SCN cells in an intact organotypic slice were tightly synchronized and exhibited stochastic rhythms comparable to those seen in the Bmal1−/− SCN explants using luminometry. Separately, we cultured dissociated SCN neurons and imaged bioluminescence from individual cells. In contrast to the cells in an intact organotypic slice, dissociated Bmal1−/− neurons did not express detectable circadian rhythms (Figure 2D, 2E, 2F, Video S2, Datasets S5 and S6). A total of 243 out of 243 Bmal1−/− SCN cells were equal to or below a threshold FFT amplitude for rhythmicity independently derived from WT SCN cells. Although Bmal1−/− SCN cells did not generate obvious circadian oscillations, they did express fluctuating levels of transcriptional noise (Figure 2F). The lack of rhythmicity in Bmal1−/− cells. Although PER1−/− cells did not generate obvious circadian oscillations, they did express fluctuating levels of transcriptional noise (Figure 2F). The lack of rhythmicity in dissociated SCN cells indicates that the stochastic rhythmicity observed in Bmal1−/− SCN explants is not a cell-autonomous property and likely arises from intercellular network interactions (to be addressed later). Thus, the integrity of the cellular anatomy of the SCN is an important factor for stochastic rhythm generation in Bmal1−/− SCN explants.

Stochastic Model of the Cell-Autonomous Circadian Oscillator

To explore possible origins of the stochastic rhythmicity in the Bmal1−/− SCN, we used mathematical modeling of circadian oscillators to examine the role of intercellular coupling and molecular noise. The key clock components in our mathematical model and their possible interactions within a single SCN cell are depicted in Figure 3A. This model is similar to the Forger-Peskin stochastic model [34] but with three refinements. In the current simulation, we (1) explicitly modeled the binding of CLOCK: BMAL to regulatory regions of the Per1, Per2, Cry1, and Cry2 genes; (2) explicitly modeled the interaction of BMAL with CRY1 or CRY2 and its subsequent effects on transcriptional regulation; and (3) updated the rates of degradation of mRNAs and proteins using measurements from recent experiments [41]. An explanation of these changes, as well as a list of rate constants, can be found in Figure S7 (also see Protocols S1 and S2).

We accounted for the heterogeneity of circadian period of SCN neurons by choosing all rate constants from Gaussian distributions. When no Gaussian variation is introduced to the rate constants (Figure 3B, “0%”), the standard deviation for period of the population of oscillators is 1.03 h, which reflects variability in period due to the stochastic model (i.e., no rate constant variability). As the standard deviation of the rate constants is increased, the standard deviation of the circadian period from a population of oscillators increases further (Figure 3B). When we chose the standard deviation of these Gaussians to be within 5% of their mean values, the resulting period of the model simulations had a standard deviation (SD = 1.54 h) similar to the experimental data obtained from dispersed WT SCN neurons, which have a standard deviation of 1.92 h for circadian period (Figure 3C). When these individual neurons were coupled in a network of cells (see below), the total mRNA and protein concentrations followed time courses similar to those found experimentally (Figure 3D).

This stochastic model was then used to simulate Bmal1−/− SCN isolated cells and the Bmal1−/− coupled SCN network, respectively. As a first step, we examined the effects of reducing the total BMAL activator concentration on the generation of rhythms in simulations of isolated cells. As the BMAL activator abundance was gradually decreased, the distribution of periods widened and the mean period shortened until a complete loss of rhythms was observed below 20% BMAL activator abundance in a population of uncoupled cells (Figure 4A and 4B). This behavior is consistent with bifurcation diagrams of single cell simulations (Figure 4C) that show the existence of a Hopf bifurcation at approximately 22% of total BMAL. Above this percentage, oscillations can be shown to emerge in our simulations. At 0% total BMAL activator abundance no element in the model could initiate transcription; thus, some level of residual BMAL activator must be present in the model in order for clock gene expression to occur.

In Bmal1 null mutants, other functional E-box activator proteins may be present. The most likely candidate for “residual BMAL” activity in Bmal1 mutants is Bmal2 (also known as MOP9; RefSeq: Arntl2 NM_172309), a paralog of BMAL1, which is regionally co-expressed in the SCN and forms transcriptionally active complexes with CLOCK [7]. Bmal1 and Bmal2 show similar sensitivity to CRY1-mediated transcriptional repression (Figure S1; for methods, see Text S1), which suggests that the core negative feedback loop of the circadian oscillator could remain functional in the presence of Bmal2. Using quantitative PCR methods that normalize the amplification of Bmal1 and Bmal2 RNA, we estimate that Bmal2 mRNA levels are approximately 10% of the level of Bmal1 and that Bmal2 expression levels are unaffected by Bmal1 loss-of-function (i.e., no compensation of Bmal2 in Bmal1−/− mutants) (Figure S2; for methods, see Text S1). Indeed recent work has shown that Bmal2 can substitute for Bmal1 if ectopically driven at high levels in Bmal1−/− mice [42]. Since Bmal1 and Bmal2 appear to be functionally equivalent, we use the term “BMAL” in our simulations to represent the sum total of Bmal1 and Bmal2.

On the basis of the expression analysis, we chose the 10% BMAL activator concentration to simulate the Bmal1−/− SCN neuron because Bmal2 represents approximately 10% of the wild-type level of Bmal1, and in Bmal1−/− cells, the remaining Bmal2 would contribute about 10% of the total Bmal1 and Bmal2 activity. Our simulated and experimentally isolated cell rhythms
Figure 2. Stochastic rhythmity in Bmal1−/− SCN is not cell autonomous. (A) Bioluminescence images of a Bmal1−/− SCN explant culture at peak and trough phases. Numbers indicate hours after start of imaging. Scale bar = 500 μm. Imaging experiments were initiated after 2–3 wk of culture. (B) Average bioluminescence and heatmap plots of bioluminescence intensity of individual Bmal1−/− neurons in an intact organotypic SCN slice. Forty cells are presented, with each horizontal line representing a single cell. These cells show tightly synchronized stochastic rhythms that are comparable to rhythms seen with PMT luminometry. (C) PER2::LUC rhythms (top) and corresponding FFT Spectrograms (bottom) for first 4 cells shown in (B) (i.e., coupled in Bmal1−/− SCN explant). (D) Bioluminescence images of dissociated individual Bmal1−/− SCN neurons showing nonrhythmic bioluminescence patterns. Numbers and scale bar are as in (A). (E) Average bioluminescence and heatmap plots of bioluminescence intensity of 40 individual Bmal1−/− neurons in dispersed culture imaged in (D), showing a lack of co-ordinated rhythmity. (F) PER2::LUC rhythms (top) and corresponding FFT spectrograms (bottom) for first 4 cells shown in (E) (i.e., dispersed Bmal1−/− SCN cells).

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Figure 3. Illustration of the elements of the stochastic model of the cellular circadian clock and heterogeneity in period length. (A) The biochemical processes modeled in an SCN cell. The specific equations and rates are included in Figure S7 (see also Protocol S1). Gray components represent molecules that could be bound but have no effect on the indicated process. This model is based on the Forger-Peskin model [14] but contains three major improvements: (1) it allows for binding and unbinding of CLOCK:BMAL to Per1, Per2, Cry1, and Cry2 genes; (2) it allows interaction of CLOCK:BMAL with CRY1 or CRY2; and (3) it uses updated rates of degradation of mRNAs and proteins measured empirically by Siepka et al. [41]. (B) Histograms of average period length of 250 simulated single cells when the variation in biochemical parameters is within 0%, 5%, 10%, 15%, 20%, or 25% of the mean values of the rate constants. (C) Histogram of single-cell periods experimentally measured from dissociated WT SCN cells. The mean and standard deviation values from this experiment were used to select the best match of mean and standard deviation for the simulated cells; the simulated values were comparable to experimental values at 5% variation in rate constants. (D) Representative traces of relative concentration of Cry1/2 and Per1/2 mRNA and protein. Circles indicate experimental data from Lee et al. [11], and lines are stochastic simulations of a population of 100 cells using the model summarized in Figure 5A. Orange and pink traces represent mRNA levels (mRNA values for Cry2 were not reported in the original data). Brown and red traces represent protein levels. doi:10.1371/journal.pbio.1000513.g003
Figure 4. Effects of BMAL activator level on circadian oscillations in a stochastic model of isolated cellular oscillators. (A) Histograms of simulated isolated cell mean period lengths at various percentages of total WT BMAL. These results show that as the percentage of total BMAL decreases, the mean period length decreases, along with an increase in the variance of the period. (B) The figure shows that as we go below certain percentage of total BMAL, rhythms in a population of uncoupled single cells disappear. This figure is an alternative way to observe bifurcations by plotting the period from a population of single un-coupled cells as a function of total BMAL. Below ~20% of total BMAL, rhythms disappear in single cells, indicating a Hopf bifurcation at this point. (C) The bifurcation diagram of a single oscillator as a function of total BMAL using a deterministic model. The y-axis plots the value G, which mathematically represents the fraction of time an E-box is activated. This was chosen since this variable affects basically all parts of the model (in particular PER1, PER2 CRY1, CRY2, all their relevant complexes, and the coupling factor). In theory, any possible variable could be used for the bifurcation diagram and the same behavior (i.e., a Hopf bifurcation) would be observed. Plotted on this diagram are the minimum and maximum (red and blue, respectively) values from the oscillation of G at a particular value of total BMAL—100% BMAL corresponds to WT BMAL. When these values are equal, the system is at rest and no oscillations are present; however, as these values begin to
show remarkable agreement at the cell autonomous level. In Figure 4D, we show representative experimental records from dissociated WT SCN cells (first row) along with their associated spectrograms (second row). Similarly, the third and fourth rows in parallel display the computational simulations for the isolated WT SCN neurons with their associated spectrograms. Comparable traces are shown for isolated Bmal1−/− cells in Figure 4E. Thus, the 10% BMAL activator simulations faithfully capture the stochastic behavior of PER2::LUC expression seen in dissociated Bmal1−/− SCN neurons.

Effects of Intercellular Coupling on Stochastic versus Deterministic Models of the Oscillator

Although there are many mathematical models of the SCN that incorporate coupling of a population of circadian oscillators [16,19–21], very few models have studied coupling in a population of oscillators in which the individual oscillators are stochastic in nature [26,27]. The results in this paper represent the most detailed stochastic simulation of coupled SCN oscillators to date. To model a population of stochastic oscillators in the SCN, we utilized a group of 100 “cell autonomous” stochastic oscillators as described in the previous section and coupled the population of oscillators (see Figures 5, S3, and S4). For the coupling pathway, we used a model of the VIP signaling pathway in the SCN, whereby a rhythmic coupling agent (CA) is released by one SCN cell and affects neighboring SCN cells by activating an adenosine 3′,5′-monophosphate (cAMP) signaling pathway, which ultimately activates cAMP response element binding (CREB) protein and cAMP response elements (CRE) on the Per1 and Per2 promoters to induce PER1 and PER2 proteins. In our proposed model, the intercellular coupling is all-to-all. We assumed that CRE activation of Per1 and Per2 could occur only when repressors were not bound as suggested in previous experiments [43]. Thus, this is the equivalent of allowing CRY to repress CREB-mediated PER production.

When coupling is included in a population of WT single-cell models, this coupled cellular network was able to produce coherent ~24-h rhythms (Figure 5B, top, and 5C, bottom) as seen in WT SCN explants and in other coupled oscillator models of the SCN [16,19–21]. Remarkably, as seen in experiments, rhythmicity also emerged when a population of nonrhythmic Bmal1−/− SCN simulated neurons was coupled together (Figure 5C, top and middle). To compare these rhythms to those found experimentally, we calculated the peak-to-peak intervals of the rhythms from the SCN network with 100%, 20%, and 10% BMAL activator concentrations (Figure 3B). The distribution of these inter-peak intervals is similar to experimental measurements from WT and Bmal1−/− explant rhythms (Figure 5B; compare with Figure 1E). The observed decrease in period in the simulated SCN explants with lower activator concentrations is similar to that observed in simulated single cells, implying that the short period of the Bmal1−/− explant may be attributable to a shortened intracellular, single-cell periodicity. However, given the general absence of rhythms in individual uncoupled Bmal1−/− cells, it is interesting that coupling of these intrinsically nonrhythmic cells (10% BMAL) can generate stochastic rhythms.

While only three sets of simulations are shown in Figure 5B and 5C, we systematically varied the coupling strength and BMAL concentration to determine the amplitude of rhythms and synchrony of the network in Figure S3. This shows that the stochastic rhythmicity can be seen over a wide range of values for the coupling strength. In addition, we note that the relative amplitudes of the Bmal1−/− simulations are reduced when compared with those found experimentally. Figure S3 shows that the amplitude of these rhythms depends greatly on the coupling strength and mechanism. In particular as the coupling becomes nonlinear, the amplitude increases. Including other nonlinear aspects of the coupling (e.g., electrical activity) could also increase the amplitude. In addition, there are likely to be other transcriptional inputs to the Per and Cry genes that could affect amplitude and that were not included in the model.

Since the emergent rhythms in the Bmal1−/− SCN explants were stochastic, we hypothesized that the molecular noise, inherent in all biochemical reactions and included in our current model through our use of the Gillespie stochastic simulation algorithm, could play an important role. To test this hypothesis, we developed a deterministic version of our proposed model to understand the implications of the noise. The deterministic model represents a version of our proposed model without any noise. Each cell in the deterministic model had its parameters chosen from a distribution as was done in the stochastic model. Once the parameter was chosen it remained fixed for the rest of the simulation. For example, the bifurcation analysis in Figure 4C used the deterministic model with its mean parameters. The stochastic and deterministic models produced equivalent results when the number of each molecule species (Figure 3D) in the stochastic model was increased. This equivalence serves as a check on the accuracy of both the stochastic and deterministic simulation approaches. The output of 100 simulated cells is shown using both the stochastic and deterministic models in Figure 5C and 5E. The total BMAL concentration was varied from 10% to 20% and finally 100% of the activator level in WT SCN, and the ensemble averages from each simulation are plotted in yellow. Strikingly, when a population of cells using the deterministic model was coupled, rhythmicity could not be recovered at 10% BMAL activator levels and only damped rhythms could be seen at 20% BMAL activator levels (Figure 5E).

The discrepancy between the stochastic and deterministic models clearly indicates that noise is a necessary but not sufficient condition for the rhythmicity observed in the Bmal1−/− SCN
and reversibly blocking voltage-dependent Na rhythms (Figure 6). TTX prevents action potentials by selectively coupling by removing TTX (Figure 6D). Our simulation results experiments that blocked coupling via TTX and then restored the preventing the sustainment of rhythmicity at the single-cell level.

Thus, TTX treatment abolished stochastic oscillations by PER2::LUC patterns similar to those of SCN neurons in dispersed caused a loss of stochastic rhythmicity at the single-cell level with WT BMAL. These results indicate that neither noise alone nor coupling alone is sufficient to produce rhythms in a population of BMAL-deficient cells. In addition, it is evident that coupling elevates the average number of PER molecules.

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Uncoupling Abolishes the Stochastic Rhythms in Bmal1−/− SCN Slices

The coupled stochastic model of the Bmal1−/− SCN predicts that both noise and coupling are required to generate stochastic rhythms. To test the role of coupling in SCN explants, we used a variety of agents previously reported to uncouple SCN neurons. We first used tetrodotoxin (TTX) treatment on Bmal1−/− SCN rhythms (Figure 6). TTX prevents action potentials by selectively and reversibly blocking voltage-dependent Na+ channels. TTX application has been shown to desynchronize neurons within an intact SCN [44], suggesting that action potentials and/or consequent neuronal transmission are required for maintaining SCN synchrony. Under continuous TTX administration, WT SCN tissue showed persistent PER2::LUC rhythms. However, the amplitude of the rhythm gradually diminished cycle by cycle (damping), which is attributable to intercellular desynchrony and a reduction in amplitude at the individual oscillator level [44]. In contrast, the Bmal1−/− SCN exhibited an immediate loss of stochastic rhythmicity of PER2::LUC output when treated with TTX. The PER2::LUC rhythms returned immediately upon removal of TTX in both WT and Bmal1−/− SCN explants (Figures 6B and S5). To determine whether the loss of stochastic rhythmicity was due to desynchronization of individual cellular rhythms, we used bioluminescence imaging to measure single-cell behavior during TTX treatment (Figures 6C and S5). Uncoupling cells by TTX treatment within an organotypic Bmal1−/− SCN slice caused a loss of stochastic rhythmicity at the single-cell level with PER2::LUC patterns similar to those of SCN neurons in dispersed culture. Thus, TTX treatment abolished stochastic oscillations by preventing the sustainment of rhythmicity at the single-cell level.

Using the coupled stochastic model of the SCN, we mimicked the experiments that blocked coupling via TTX and then restored the coupling by removing TTX (Figure 6D). Our simulation results reproduce the experimental data for both the WT and Bmal1−/− SCNs; these results demonstrate that abolition of coupling alone in the model can account for the observed patterns of PER2::LUC bioluminescence from Bmal1−/− SCN when blocked with TTX.

Similar findings were observed when other pharmacological agents were used to uncouple the SCN neurons (Figure S6). For example, pertussis toxin treatment, which blocks Gαs-mediated signal transduction, and bicuculline, which blocks GABA_A receptors, both abolished PER2::LUC stochastic rhythmicity—these agents have previously been shown to uncouple SCN cells [45,46].

Role of the cAMP and PER Induction Pathway in Coupling and Period Determination

A prominent pathway for coupling SCN neurons involves the VIP receptor-signaling pathway that activates cAMP and CREB-mediated induction of Per1 and Per2 transcription [37,38,43,47,48]. To test the role of the cAMP signaling pathway in coupling in SCN explants, we used MDL-12,330A (MDL), a potent inhibitor of adenyl cyclase, and H-89, an inhibitor of cAMP-activated protein kinase (protein kinase A; PKA) (Figure 7). Previous studies have shown that MDL reduces cAMP concentrations to basal levels in SCN cells [47], and H-89 prevents VIP- or calcium-induced circadian transcription in SCN cells [49]. Similar to the effect of TTX treatment, WT SCN explants showed damped rhythms with prolonged exposure to these inhibitors of cAMP-mediated signal transduction (Figure 7B and 7C, left), and the Bmal1−/− SCN explants showed an immediate loss of stochastic rhythmicity (Figure 7B and 7C, right). Thus, the cAMP pathway appears to be necessary for the generation of stochastic oscillations.

In the circadian clock mechanism, the half-life of the PER proteins contributes to the determination of circadian period, which is under the control of CK1ε/δ-mediated PER phosphorylation [50–52]. Modeling studies have also suggested that the Per2 feedback loop, in particular, may have a dominant role in setting the period of circadian oscillation [53]. The VIP/cAMP signaling pathway ultimately converges on Per induction, and thus the periodicity of stochastic rhythms in Bmal1-null mutant SCN may also depend upon PER. To test a role for PER proteins in determining the period of stochastic rhythms, we examined the effects of agents that inhibit CK1ε/δ to lengthen circadian period. We treated SCN explants with a potent protein kinase inhibitor, SP600125, which has been shown to lengthen circadian period and to inhibit CK1ε kinase activity [51]. SP600125 treatment lengthened WT SCN circadian period to over 30 h (Figure 8A, top and 8B, left) as shown previously. Interestingly, SP600125 also significantly lengthened the period (average inter-peak intervals) of the stochastic rhythms from Bmal1−/− SCN explants (Figure 8A, bottom and 8B, right). Modeling experiments in which the phosphorylation
rate of PER by CK1 was reduced also lengthened the periodicity of WT and Bmal1−/− mutant SCN simulations in a similar fashion (Figure 8C). Thus, the kinetics of the quasi-circadian periodicity that emerges from the SCN network are regulated by a PER-dependent process in which the phosphorylation rate of PER by CK1 can determine the periodicity of the emergent network oscillation.

**Discussion**

The circadian pacemaker in the SCN plays a dominant role in the generation and control of circadian behavioral rhythms in mammals [54,55]. While it is well established that the generation of circadian rhythmicity is a cell-autonomous property of SCN neurons [1] and that coupling plays an important role in...
enhancing the precision of circadian oscillations [56], it has not previously been demonstrated that the SCN network itself, in the absence of cell autonomous oscillatory function, can generate quasi-circadian oscillations. The experiments reported here argue strongly that a PER-dependent neural coupling mechanism in the SCN can provide a feed-forward signal to drive the circadian

Figure 7. Inhibition of cAMP signaling abolishes stochastic rhythms from Bmal1−/− SCN explants. (A–C) Representative records of PER2::LUC rhythms of the SCN explants from WT (blue) and Bmal1−/− (red) mice. Data are shown following a medium change (day 8); shaded area indicates when SCN explants were chronically treated with vehicle solution (A), 1 μM MDL (B), or 10 μM H-89 (C). WT SCN explants recovered rhythms immediately following the washout of MDL (B, left) or H-89 (C, left); however, almost all Bmal1−/− explants required second washout to reinitiate their rhythms (B and C; right).
doi:10.1371/journal.pbio.1000513.g007
and propagate quasi-circadian oscillations under conditions when cell-autonomous circadian oscillations have been abolished by the loss of Bmal1 function. Mathematical modeling experiments comparing stochastic and deterministic models of the coupled SCN network strongly suggest that both molecular noise and intercellular coupling are essential for the generation of “emergent” oscillations from the network.

While it is clear that coupling is essential for normal SCN function [37,38,44] and is responsible for the robustness of SCN oscillations [28,56], there are no precedents demonstrating that coupling per se can generate oscillations in the circadian domain. In the field of central pattern generators, rhythmic outputs can be generated by circuits that are devoid of intrinsically rhythmic pacemaker neurons [57]. In such cases, rhythmicity is an emergent property of the network. The time course of central pattern generators, however, is orders of magnitude faster than circadian oscillations. Thus, it seems likely that the mechanisms leading to oscillations in these two time domains will differ. This indeed appears to be the case since transcriptional rather than synaptic mechanisms underlie the long time constants in the circadian domain.

Figure 8. Effects of SP600125 on periodicity in WT and Bmal1−/− SCN explants. (A) Representative records of PER2::LUC rhythms from SCN explants of WT (top) and Bmal1−/− (bottom) mice. Shown are 8 d of bioluminescence record before SP600125 treatment followed by 6 d of bioluminescence record during the SP600125 (25 μM) treatment (shaded). At the time indicated by orange arrow, individual SCN explants were changed into a fresh medium with the kinase inhibitor. (B) Average inter-peak intervals of PER2::LUC rhythms (±SEM) of WT (left; before = 24.86 ± 0.19 h, SP600125 = 32.65 ± 0.43 h) and Bmal1−/− (right; before = 17.92 ± 0.60 h, SP600125 = 20.32 ± 0.42 h) SCN explants. SP600125 treatment lengthened the intervals in both WT (paired t-test, p < 0.0001, df = 5) and Bmal1−/− (paired t-test, p < 0.0005, df = 6) SCN explants. (C) Average period (±SEM; n = 6 per data point) from simulated WT (blue) and Bmal1−/− (red) SCN networks with varying percent inhibition of PER phosphorylation by CK1. The error bars (±SEM) cannot be seen in some of the data plots because the symbol is larger than the bar. Period values of WT and Bmal1−/− SCN networks were significantly different from each other and as a function of inhibition of PER phosphorylation (two-way ANOVA, p < 0.001).

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Because the mammalian circadian clock mechanism involves gene expression, and gene expression is affected by molecular noise [58–60], noise is expected to influence circadian clocks. Indeed molecular noise is a key contributor to the stochastic nature of intracellular rhythm [61]; and overcoming molecular noise has been proposed as a key principle in the design of circadian clocks [35]. However, in these previous examples, reduction rather than enhancement of noise has been the key factor. A novel finding of the present study is that molecular noise can be an integral part of the functional SCN network. Molecular noise is amplified by a sensitive coupling mechanism among SCN neurons and can “kick start” oscillations within the network. Future modeling work, using more detailed models of electrical [62,63] and chemical [16,21,64] signaling in the SCN, can be used to identify the specific nonlinearities that can cause noise-induced oscillations [17]. The highly sensitive nature of the coupling mechanism allows for the amplification of not only the oscillatory signals but also the noise intrinsic in the overall network. This noise amplification can explain why the rhythms in Bmal1−/− SCNs are highly variable. Therefore, sensitive transcriptional regulation mechanisms that play a crucial role in promoting rhythmicity are also likely to amplify the effects of molecular noise. This finding is consistent with previous studies, indicating how certain choices of transcriptional regulatory mechanisms can greatly amplify the effects of molecular noise thereby leading to noise-induced oscillations [31–35].

Our simulations were able to compare mathematical models with and without noise, something that cannot be easily accomplished experimentally at this time. Molecular noise depends on the number of reactions that take place in a system. Although future experimental techniques may be able to address this issue, they do not exist for the larger mammalian genetic networks [65]. Also, stochastic bifurcation theory could be applied to understand our numerical results mathematically.

Classic work has shown that noise can induce oscillations [66]. In addition, there is a growing body of literature that demonstrates how molecular noise can enhance the behavior of cellular networks within many organisms (reviewed in [58]). Similar theoretical and experimental studies of the somite segmentation clock in vertebrate development have shown how coupling can be used to reduce the effects of noise [67,68]. Another recent modeling paper has shown how coupling and noise can synergistically enhance calcium oscillations in two coupled cells [69]. This does not mean that noise is beneficial in all circumstances. However, we demonstrate here that the effects of molecular noise can propagate in a large network of coupled cells and may even be beneficial. This result is counterintuitive since previous work indicates that coupling diminishes the effects of noise as one increases the number of cells in the network [17]. Thus, our work establishes the importance of molecular noise in the functioning of intercellular networks. The emergence of stochastic circadian oscillations from the SCN network in the absence of cell-autonomous circadian oscillatory function highlights a previously unrecognized level of circadian organization. While we acknowledge that it is difficult to assess the role of such noise-induced oscillations in the wild-type animal in which oscillations from the cell autonomous oscillators would be expected to dominate the ensemble output from the SCN network, the resonance in the coupling network highlighted here could contribute to the robustness of the mammalian circadian system. In addition, network oscillations of the type reported here could also underlie other rhythmic phenomena such as the food-entrainable oscillator or the methamphetamine-inducible oscillator, which are thought to be independent of the circadian pacemaking system in the SCN [70–74]. These normally occult oscillators appear to require either restricted food availability or psychostimulant drugs to activate or reveal their presence. Perhaps feed-forward inputs from feeding signals or psychostimulants can generate quasi-circadian oscillations in behavior in a manner analogous to the stochastic SCN network oscillations reported here.

Materials and Methods

Animals and Behavioral Analysis

All animal care and experimental treatments were approved and performed in strict accordance with Northwestern University and University of Texas Southwestern Medical Center guidelines for animal care and use. Mice were bred from PER2::LUC [http://jaxmice.jax.org/strain/006852.html] homozygous parents [4] mated with Bmal1+/− mice [4,7]. From the first generation of pups, mice carrying a copy of the Per2:Luc (lac+/-) and heterozygous for the mutation in Bmal1 were then crossed to obtain Bmal1+/−, Bmal1+/−, and Bmal1−/− mutants harboring the PER2::LUC reporter.

At approximately 8–10 wk of age, mice were placed in individual running wheel cages, and activity was recorded using the ClockLab data collection system (Actimetrics, Wilmette, IL) [75]. After 2 wk in LD 12:12, the mice were released into constant darkness (DD) for an additional 4 wk. Animals were then returned to LD 12:12 for at least 2 wk before their tissues were harvested for bioluminescence experiments.

SCN Explant Culture and Bioluminescence Data Analysis

Animals were euthanized by cervical dislocation between ZT 11–13. The tissues were removed immediately and put in Hank balanced salt solution (HBSS; with 10 nM HEPES, 25 units/ml penicillin, and 25 µg/ml streptomycin) on ice. Brain slices containing the SCN were sectioned at 300 µm using a vibratome followed by scalp dissection of the SCN, resulting in a piece of tissue about 1 mm x 1 mm in size. The peripheral tissues were dissected into pieces approximately 1 mm² in size, with the exception of the pituitary, which was cultured as a whole. Each dissected tissue was cultured on a Millicell culture membrane (PIMORG50, Millipore) with 1.2 ml DMEM medium (Cellgro), supplemented with 10 mM HEPES (pH 7.2), 2% B27 (Invitrogen), 25 units/ml penicillin, 25 µg/ml streptomycin, and 0.1 mM luciferin (Promega) as described previously [76]. Medium changes to LD 12:12 for at least 2 wk before their tissues were harvested for bioluminescence experiments. The tissues were maintained at 36 °C either in an incubator or in a temperature-controlled room. The bioluminescence was continuously monitored with LumiCycle photomultiplier tube (PMT) detector systems (Actimetrics, Wilmette, IL). Dark counts from the PMT were measured with luciferin-containing medium alone and subtracted from overall bioluminescence. Bioluminescence was measured immediately upon placement in culture, continuing without interruption for >7 d.

Bioluminescence analyses were performed using the LumiCycle Analysis Program (Actimetrics, Wilmette, IL). Raw data were
observed between WT and stability of average daily bioluminescence, and no differences were 7 d. SCN neuron viability was assessed by cell morphology and 29.8 min exposure duration were collected at 30 min intervals for noise ratio was improved by 2
m
400
10-d-old pups, cut by tissue chopper (Stoelting) to a thickness of neurons were maintained in culture for 2–7 wk before imaging. For TTX experiments, adult SCN explants were dissected as described in the previous section. For preparation of dispersed SCN neurons, we used 2–5-d-old
Bmal1
−/− pups (or WT controls from the same heterozygous breeding line). Cylindrical punches of unilateral SCN were made from 400 μm coronal sections using a 20-gauge needle. Punches from 2–6 mice were pooled in each preparation, and the experiment was performed twice for each genotype. Cells were dissociated using papain and cultured as previously described [1], except that medium contained 5% FBS instead of rat serum. SCN were dissected as described in the previous section. From 2–6 mice were pooled in each preparation, and the experiment was performed twice for each genotype. Cells were dissociated using papain and cultured as previously described [1], except that medium contained 5% FBS instead of rat serum. SCN neurons were maintained in culture for 2–7 wk before imaging.

Single-Cell Imaging

Bioluminescence imaging was performed as previously reported [3,28,77]. Just before imaging, medium was changed to fresh explant medium containing 1 mM luciferin. Culture dishes were sealed and placed on the stage of an inverted microscope (Olympus IX71) in a dark room. A heated lucite chamber around the microscope stage (Solenet Scientific, UK) kept the cells at a constant 36 °C. For experiments presented in Figure 2 and Videos S1 and S2, images were collected using an Olympus 4× XLFLUOR (NA 0.28) or UPlanSapo (NA 0.16) objective and transmitted to a CCD camera (Spectral Instruments SII800, Tucson, AZ) cooled to −92 °C. For dispersed neurons, signal-to-noise ratio was improved by 2×2 binning of pixels. Images of 29.8 min exposure duration were collected at 30 min intervals for 7 d. SCN neuron viability was assessed by cell morphology and stability of average daily bioluminescence, and no differences were observed between WT and
Bmal1
−/− cells. For TTX experiments, adult SCN slice images were collected with a dual microchannel plate intensified gallium arsenide phosphide XR/MEGA-102Z CCD camera (Stanford Photonics, Inc., Palo Alto, CA) cooled to −20 °C.

Single-Cell Imaging Data Analysis

Bioluminescence was analyzed using MetaMorph (Molecular Devices) as previously described [3,28,77]. For TTX experiments, CellCycle single-cell analysis software (Actimetrics, Wilmette, IL) was used. Cells that were clearly discriminable from adjacent cells and that remained bioluminescent for the entire experiment were selected for analysis. Bioluminescence time series were first imported into LumiCycle Analysis v. 2.31 (Actimetrics). A linear baseline fitted. Then peak-to-peak durations (inter-peak intervals) were measured by manually identifying individual peaks. Serial correlation coefficient estimates were calculated for 7 to 10 cycle epochs using methods described previously [39].

SCN Slice and Dispersed Cell Culture for Imaging

Bmal1
−/− SCN slices used for imaging were dissected from 4–10-d-old pups, cut by tissue chopper (Stoelting) to a thickness of 400 μm, and cultured on Millicell-CM membrane inserts (PICMORG50). SCN slices were maintained in culture for 2–3 wk before imaging. For TTX experiments, adult SCN explants were dissected as described in the previous section.

Mathematical Simulation

Simulations used a revised version of the Forger-Peskin stochastic model of the mammalian circadian clock [14,34]. We explicitly modeled the binding and unbinding of CRY1 or CRY2 (and any other bound proteins) with CLOCK:BMAL (which has the same biochemical properties as the CLOCK:BMAL1 or CLOCK:BMAL2 complexes, which were treated equivalently). Based on experimental evidence [20,79], we assumed that the concentration of these protein complexes was constant (1,600 molecules per cell for the WT case). The original Forger-Peskin model [14] contained a binding event of the CRY proteins to activator on the promoter. This was replaced by a binding event of CLOCK:BMAL to the promoter. Consistent with fitting of experimental data [41] we assumed that there was just one active E-box on the promoters of Per1, Per2, Cry1, and Cry2. Reaction rates can be found in Figure S7.

We also modeled a CRE element where a factor (e.g., CREB) can bind and activate transcription of Per1 and Per2 or a secreted CA. The “coupling” factor is equivalent to AVP or VIP and mediates signaling between SCN neurons (see Figure 5). Since medium changes can stimulate transcription, presumably through this CRE element, we assumed that there was a constant low level of this factor in the network (one molecule per cell). Removing this constitutive activation had little effect on our simulations (unpublished data).

Due to the revised transcription mechanism, new rates of transcription were chosen to give qualitative agreement of the uncoupled model with protein concentration levels determined experimentally by Lee et al. (2001) [11]. Other parameters were fit to published experimental data on the mRNA and protein time courses used in previous models [14,34]. Degradation rates for Per1/2 and Cry1/2 mRNAs and CRY1 and PER2 proteins were obtained directly from published experimental data [41]. This yielded a model with a slightly short period (22.9 h) that was scaled to give a 24-h period.

Simulations were performed using the Gillespie Algorithm as in Forger and Peskin (2005) [34] or MATLAB’s ode15s with a maximum time step of 0.05 h. In the limit of large numbers of molecules, these simulations matched, as did a version of the model coded in Mathematica. Simulations typically lasted 500 h; the first 50–100 h were discarded to remove the effects of initial transients. Using MATLAB, spectrograms of the experimental and simulated single cell and slice data were produced. Specifically, the “spectrogram” command was used, with window size 128, overlap set to 100, and sampling set to 48 cycles per day. Simulated data were collected and analyzed in the same manner as the experimental data (Figure 4D, 4E). Network simulations contained 100 cells with mean field coupling. We then calculated the normalized amplitude and the order parameter defined by Garcia-Ojalvo and colleagues [80]. Codes written in the C programming language are available as Protocol S1.

Supporting Information

Dataset S1 Bioluminescence data from 14 individual WT SCN explants.
Found at: doi:10.1371/journal.pbio.1000513.s001 (0.68 MB XLS)

**Dataset S2** Bioluminescence data from 16 individual Bmal1^−/− SCN explants.
Found at: doi:10.1371/journal.pbio.1000513.s002 (0.86 MB XLS)

**Dataset S3** Bioluminescence data from 40 individual Bmal1^−/− SCN cells in an intact SCN explant slice.
Found at: doi:10.1371/journal.pbio.1000513.s003 (0.31 MB XLS)

**Dataset S4** Bioluminescence data from 40 individual Bmal1^−/− SCN cells in an intact SCN explant slice.
Found at: doi:10.1371/journal.pbio.1000513.s004 (0.29 MB XLS)

**Dataset S5** Bioluminescence data from 115 individual Bmal1^−/− SCN cells dispersed in culture.
Found at: doi:10.1371/journal.pbio.1000513.s005 (2.59 MB XLS)

**Dataset S6** Bioluminescence data from 128 individual Bmal1^−/− SCN cells dispersed in culture.
Found at: doi:10.1371/journal.pbio.1000513.s006 (4.72 MB XLS)

**Figure S1** BMAL1 and BMAL2 transcriptional activity show similar sensitivity to CRY1 repression. Sensitivity to CRY1 repression was tested by comparing the activities of CLOCK::BMAL1 or CLOCK::BMAL2 complexes in the presence of 0 to 25 ng of Cry1 plasmid in Per1-luciferase reporter assays (as described in [81]). BMAL1 and BMAL2 show similar sensitivity to CRY1-mediated transcriptional repression, suggesting that BMAL2 may account for a low level of activation in the absence of BMAL1. Bmal2-13, -14, and -15 represent three independent Bmal2 clones. For the reporter assay, 293T cells were transiently transfected with Lipofectamine 2000 (Invitrogen) containing 100 ng Bmal1 or Bmal2 construct, 100 ng Clock, 20 ng Per1-Luc reporter, and increasing amounts of Cry1.
Found at: doi:10.1371/journal.pbio.1000513.s007 (0.30 MB TIF)

**Figure S2** Relative Bmal1 and Bmal2 expression levels in WT and Bmal1^−/− tissues – Bmal1 and Bmal2 mRNA levels were measured using quantitative PCR. The primers were designed and tested to have consistent amplification efficiency for both Bmal1 and Bmal2 template cDNAs. Tissues were collected from animals housed under a light:dark cycle (12 h light:12 h dark) at three different time points (zeitgeber time 12, or ZT 12 = onset of dark period). In the case of Bmal1^−/− tissues, the mutant transcript level was measured and displayed in the graph. In WT tissues, Bmal1 clearly shows daily oscillations peaking at ZT10 in the brain and at ZT12 in the liver; however, in Bmal1^−/− tissues, the mutant transcript levels are elevated throughout the day and do not show oscillations (presumably due to the absence of Rev-erb-alpha repression of Bmal1). The total level of Bmal2 expression is about 10% of the average daily level of Bmal1 in WT tissues, and its expression is unaffected by Bmal1 loss-of-function (in Bmal1^−/− tissues).
Found at: doi:10.1371/journal.pbio.1000513.s008 (0.75 MB TIF)

**Figure S3** PER2 levels from simulated cells with varying amounts of BMAL and different coupling mechanisms. Coupling is critical to understanding the generation of stochastic oscillations in the presence of noise. Therefore, we explored the effectiveness of our hypothesized coupling mechanism in our proposed network model (see Figure 3A). Specifically, we studied the amplitude and order parameter (a measure of the synchronization in a population of cells) as a function of the amount of activator concentration and coupling strength in a population of 100 cells. The order parameter is a mathematical quantity to measure the level of synchronization in a population of cells [80]. It is the ratio of the variance of the averaged ensemble’s PER2 (numerator) and the average variance of each individual cell’s PER2 in the ensemble (denominator). Furthermore, this parameter is a dimensionless number between 0 (no synchronization, all cells out of phase) and 1 (fully synchronized, all cells in-phase). The coupling strength is a measure of the strength of the inter-cellular coupling strength. It is implemented by changing the production rate of the coupling factor within the model. (A) Amplitude and order parameter surface plots of a population of 100 cells. At right are traces of PER2 levels of simulated cells in the network (blue) along with the population average (yellow); these traces were simulated using the amounts of BMAL and intercellular coupling that produced the strongest amplitude. The specific values for BMAL and intercellular coupling are BMAL = 1.0, coupling = 0.015. Based on these plots, synchronization occurs over a wide range of parameter values, and therefore the systems behavior is not sensitive to this value. (B) Additional simulations at various ratios of BMAL and coupling strength as noted in individual plots. These values were selected to represent the strongest coupling strength with the lowest BMAL ratio, strongest coupling strength with the highest BMAL ratio, the weakest coupling with the lowest BMAL ratio, the weakest coupling with the highest BMAL ratio, and a level in between. In all simulations, indicated in this figure, the same coupling mechanism is used. We observe that as the strength of the coupling increases in the network, the amplitude of the emergent oscillations also increases.
Found at: doi:10.1371/journal.pbio.1000513.s009 (0.87 MB TIF)

**Figure S4** Phase portraits of simulated Per2 mRNA and simulated PER2 protein. (A) Phase portraits of PER2 protein and Per2 mRNA from both WT and Bmal1^−/− simulations of SCN network. We plotted Per2 mRNA against PER2 protein from a WT or Bmal1^−/− SCN network simulation. Graphs that are plotted in this manner are called phase portraits, which are geometric trajectories of the dynamical system. In the WT SCN (left panel), we observe a limit cycle in the plane indicative of stable oscillations. Here the phase portraits reveal sustained oscillations, as indicated by the limit cycle in this figure. On the right panel, we plotted Per2 mRNA against PER2 protein from a Bmal1^−/− network simulation. The phase portraits in Bmal1^−/− do not reveal clear limit cycles; this fact does not rule out oscillatory behavior and may indicate instead the existence of stochastic behavior that is aided by the existence of noise in the network. (B) WT and Bmal1^−/− SCN network simulations of PER2 protein and Per2 mRNA as a function of time. In the left panel, we see the changes in WT PER2 protein and Per2 mRNA concentrations as a function of time. As expected, these profiles show oscillations that are approximately 24 h. However, on the right panel, we observe that PER2 protein and Per2 mRNA concentrations from Bmal1^−/− SCN simulations have emergent but stochastic rhythms.
Found at: doi:10.1371/journal.pbio.1000513.s010 (0.59 MB TIF)

**Figure S5** PER2::LUC bioluminescence from individual Bmal1^−/− and WT SCN neurons before, during, and after TTX treatment. Individual single-cell records before, during, and after TTX treatment from cells within intact Bmal1^−/− and WT SCN slices from Figure 6C. Uncoupling cells by TTX treatment within an intact organotypic Bmal1^−/− SCN slice results in arrhythmic single-cells with PER2::LUC patterns similar to those of SCN neurons in dispersed culture (see Figure 2F). Under continuous TTX administration (red bar), WT SCN cells are able to maintain PER2::LUC rhythms, although with diminished amplitude. However, when treated with TTX, the Bmal1^−/− SCN neurons show an immediate, complete loss of rhythmicity. Upon removal of TTX, the robustness returns to the WT single-cell rhythms and the stochastic rhythmicity returns to Bmal1^−/− SCN neurons.
Found at: doi:10.1371/journal.pbio.1000513.s011 (0.65 MB TIF)
Figure S6 Uncoupling SCN cells abolishes stochastic rhythms from Bmal1−/− SCN explants. Representative records of PER2::LUC rhythms of the SCN explants from wild type (blue) and Bmal1−/− (red) mice. Data are shown following a medium change (day 8); shaded area indicates when SCN explants were changed to fresh medium containing vehicle solution (A), Tetrodotoxin (B), Bicuculline (C), and Pertussis toxin (D). (A and B) The effects of vehicle (A) and tetrodotoxin (B) treatments are discussed in the main text of the manuscript (see Figure 6). (C) The application of GABA\(_A\) receptor antagonist, bicuculline (BIC), effectively blocks GABA-evoked inhibitory postsynaptic currents preventing inter-regional synchronization between dorsal and ventral SCN [45]. Bicuculline application to WT SCN explants showed gradual damping of the PER2::LUC peak-to-trough amplitude; however, it also showed heightened overall luminescence, indicating a higher level of PER2 expression (C, left). The increase in luminescence level was also seen in Bmal1−/− SCN with BIC treatment, but the rhythmic nature of PER2::LUC was eliminated. When BIC was removed, the WT SCN rhythm was immediately restored. Bmal1−/− SCN briefly showed residual effects of BIC and took 1 to 2 d to fully recover its rhythmicity (C, right). (D) Pertussis toxin (PTX) irreversibly inactivates Gi and Go protein activity by preventing their inhibition of adenylyl cyclase; consequently, its application prevents Gi/o-mediated coupling within the SCN. PTX treatment has been shown to both decrease the synchrony among rhythmic neurons and abolishes rhythms in a subset of neurons within the SCN [46]. PTX application to WT SCN significantly reduced the peak-to-trough amplitude leading to damping of rhythms, as was observed with TTX and BIC treatments. Bmal1−/− SCN responded to PTX in a similar fashion as did to BIC treatment—the PER2::LUC stochastic rhythmicity was completely abolished with an overall increase in luminescence level. Unlike the previous two treatments that were used, rhythms did not fully return when PTX was removed in either WT or Bmal1−/− SCN, likely due to the irreversible nature of PTX. (E) Relative values of PER2::LUC luminescence brightness and FFT power for SCN explants from above (B–D). Bioluminescent counts before, during, and after each of the drug treatments were averaged to obtain Relative Brightness. Spectral analysis (FFT power spectrum, Blackman-Harris windowing) as described previously [78] was used to estimate the amplitude of the circadian frequency range using ClockLab software (Actimetrics, Wilmette, IL). Values for time intervals (±SEM) were obtained before, during, and after the treatment are graphed for each genotype and treatment. Relative brightness: there was no significant genotype effect by ANOVA, \(F(2,36) = 0.01, p > 0.16\), but significant drug effect in luminescence level, \(F(3,36) = 0.01, p < 0.00003\); Tukey-Kramer, \(p < 0.05\). Relative FFT Power: there were both significant genotype and drug effects, \(F(1,45) = 0.01, p < 0.008\), and \(F(2,36) = 0.01, p < 0.0005\), respectively; Tukey-Kramer, \(p < 0.05\). Found at: doi:10.1371/journal.pbio.1000513.s012 (0.76 MB TIF)

Figure S7 Table of reaction rates and summary of changes. Listed rate constants are for the stochastic model. Deterministic rate constants can be determined by setting \(V = 1\). Changes from the Forger and Peskin model [14] are highlighted as follows: New reactions are highlighted in green; updated parameters, based on the hall-lives reported by Siepka et al. [41], are highlighted in yellow. To match these data, we also increased the rate of formation and transport of mRNA to the cytoplasm (trn) from the slow rate used in [34] and slightly increased the rate of export of proteins from the nucleus (trp). Since a new mechanism of transcription regulation was used (where just one strong E-box drives transcription of all genes), several of our reaction rates were updated (highlighted in blue) including the maximal rate of transcription of the PER and CRY genes (trPo, trP, trRo, trR); the translation rate of PER (trP); and the concentration of the kinases (Ck) to match the data reported by Lee et al. [11] on the relative concentration of clock proteins. The new concentrations in the model can be converted to number of molecules equivalent to those reported in [34] by choosing \(V = 200\). Found at: doi:10.1371/journal.pbio.1000513.s013 (0.92 MB TIF)

Protocol S1 Code for the stochastic mathematical simulation of the circadian molecular clock; the code is written in the C programming language. This code was used to generate simulations found in Figures 3B, 4A, 4D, 4E, 5B, 5C, 5D, 5F, 6D, 5C, S3, and S4. The parameters, and a brief description, used in this code can be found in Figure S7. Found at: doi:10.1371/journal.pbio.1000513.s014 (0.05 MB DOC)

Protocol S2 Description of model equations in human readable format. The equations for this model, parameters, and a brief description can be found in Figure S7. The equations were simulated in MatLab and were used to generate Figures 3D, 4B, 4C, 5D, 5E, and 5F. Found at: doi:10.1371/journal.pbio.1000513.s015 (0.18 MB DOC)

Text S1 Supplementary methods. Found at: doi:10.1371/journal.pbio.1000513.s016 (0.06 MB DOC)

Video S1 Bioluminescence expression patterns from Bmal1−/− SCN explants. Found at: doi:10.1371/journal.pbio.1000513.s017 (1.36 MB MOV)

Video S2 Bioluminescence expression patterns from Bmal1−/− dissociated SCN neurons. Found at: doi:10.1371/journal.pbio.1000513.s018 (1.48 MB MOV)

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: CHK YRY DKW EDB ACL EEZ DBF JST. Contributed reagents/materials/analysis tools: MRR EDB ACL EEZ DBF JST. Performed the experiments: CHK YRY DKW SAK DBF JST. Analyzed the data: CHK YRY DKW EDB ACL EEZ DBF JST. Wrote the paper: CHK YRY DBF JST.

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