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Challenges in synthetically designing mammalian circadian clocks

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Synthetic biology, in which complex, dynamic biological systems are designed or reconstructed from basic biological components, can help elucidate the design principles of such systems. However, this engineering approach has only been applied to a few simple biological systems. The circadian clock is appropriate for this approach, since it is a dynamic system with complex transcriptional and post-transcriptional circuits that have been comprehensively described. Rational synthesis of the properties of the suprachiasmatic nucleus, the central clock tissue of the circadian system that controls many dynamic behaviors, will be important for understanding the neural-circuit systems that control physiological behaviors. These approaches will provide a deeper understanding of the biological clock, and of clinical problems associated with it, such as sleep disorders.

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The recent demands for integrating large-scale datasets to reach a deeper understanding of living systems has set the stage for the advent of systems and synthetic biology [1,2••]. Complex and dynamic biological phenomena, such as the circadian clock, are suitable subjects for these newly emerging approaches [2••], in which developing an understanding of a system is a four-step process: *identification*, *analysis*, *control*, and *design* of the system of interest. The components and their networks sufficient to the function of the system are confirmed in the final step, *design*, in which the original system is reconstructed from scratch, using rationally synthesized biological components, pathways, and networks. Here, we use the mammalian circadian system as an example to discuss how this synthetic approach can be applied to gain a comprehensive understanding of the systems. In engineering and therapeutic scenarios, this approach can help manufacture precise and timely interventions of the intracellular or intercellular regulatory mechanisms and rational reprogramming of cell/tissue phenotypes of the circadian clock, which will be of growing importance particularly in medical applications.

Overview of the mammalian circadian clock and application of synthetic biology

The circadian clock is an evolutionarily conserved molecular biological timing system. Its underlying mechanisms consist of intracellular auto-regulatory feedback loops in which specific proteins called *clock proteins* rhythmically activate or repress each other [2••,3••,4••,5•,6••]. Circadian clocks in multi-cellular organisms are organized as a hierarchy of circadian oscillators. Peripheral circadian clock cells are widely distributed in a variety of tissues throughout the body [2••,3••,4••,5•,6••,7•]. One central clock tissue located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus regulates the circadian rhythms of these peripheral clock cells in mammals. More specifically, the SCN orchestrates these circadian rhythms according to external cues, including light [3••,4••,6••]. The circadian clocks in central and peripheral tissues are intimately involved in the regulation of metabolic and physiologic processes. Impairment of the circadian clock is associated with numerous diseases, including sleep disorders, depression, cancer, and dementia [2••,3••].

Synthetic approaches to understanding mammalian circadian clocks can be divided into synthesis of a *molecular clock*, and synthesis of the *central clock*. The first involves the rational design of minimal artificial transcriptional and post-transcriptional networks, often these are (simplified) replicas of the original structure and function that is shared by central and peripheral clock cells. The second application involves a rational design of the properties of the SCN, the central clock tissue. This requires implementing the signal transduction network, electrophysiological network, and intercellular circuits of

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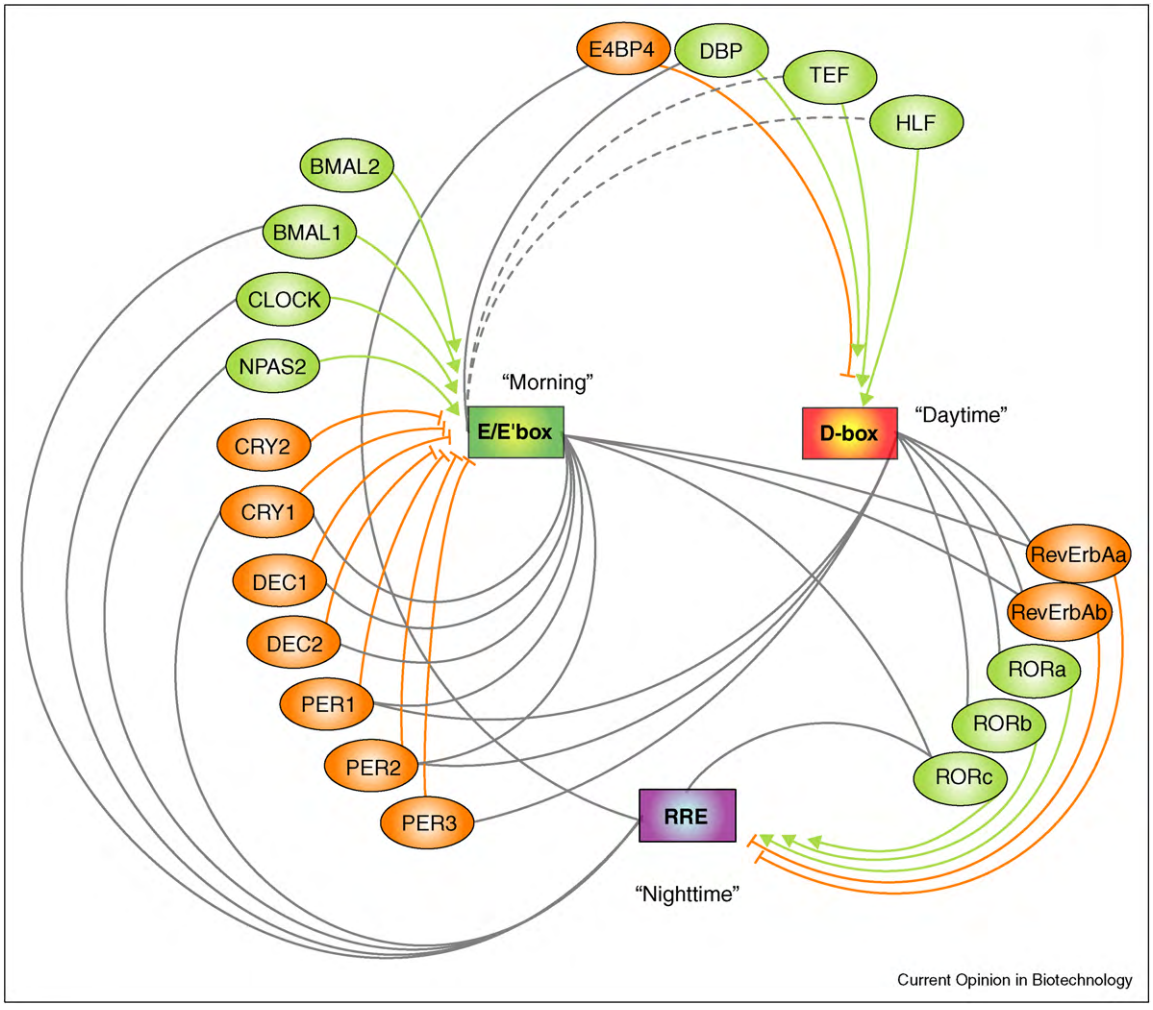
the SCN (e.g., gating, electrophysiological oscillations, and coupling) in non-SCN cells.

Transcriptional networks of mammalian circadian rhythms

The circadian system in multi-cellular organisms has well-defined dynamic properties, including: first, endogenous oscillations with an approximately 24-h period; second, entrainment to external environmental changes (temperature and light cycles) and third, temperature compensation over a wide range of temperatures. In mammalian clocks, circadian transcriptional oscillations are governed, at least in part, by transcriptional programs that rely on at least three clock-controlled *cis*-elements (CCEs): morning (E-box/E′-box, CACGT[G/T]), day-time (D-box, TTA[T/C]GTAA), and night-time (RevErbA/ROR binding element or RRE [A/T]A[A/T]NT[A/G]GGTCA) elements [2••,3••]. Many molecules controlling the circadian transcription program via the three CCEs have been reported, as described in Figure 1.

The E-box-mediated transcriptional program is critical in the core auto-regulatory loop of the mammalian circadian clock [2••,3••,5•,7•]. According to a current clock model, bHLH-PAS transcription activators such as BMAL1 and CLOCK form heterodimers that bind to E-box/E′-box *cis*-elements present in the promoter regions of their target genes, which include the *Per* and *Cry1* genes. In turn, the CRYs and PERs induced by the BMAL1/CLOCK heterodimers form repressor complexes; these physically

Figure 1



Overview of the transcriptional network of the mammalian circadian clock. Genes, CCEs, transcriptional/translational expression, activation, and repression are depicted as ovals, rectangles, gray lines, green lines, and orange lines, respectively. The E-box-mediated transcription program is directly or indirectly controlled by at least 11 transcription factors. These include four basic helix–loop–helix (bHLH)-PAS transcription activators, *Clock*, *Npas2*, *Bmal1* (also known as *Arntl* or *Mop3*), and *Bmal2*; three Period genes, *Per1*, *Per2*, and *Per3*; two Cryptochrome transcription repressors, *Cry1* and *Cry2*; and two other bHLH transcription factors, *Bhlhb2* and *Bhlhb3* (also known as *Dec1* and *Dec2*). At least four bZIP-family genes, *Dbp*, *Hlf*, *Tef* and *E4bp4* (also known as *Nfil3*), and five orphan nuclear hormone receptors, *Nr1d1*, *Nr1d2* (also known as *RevErbAα*, *RevErbAβ*), *Rora*, *Rorb* and *Rorc*, control the D-box- and RRE-mediated transcription programs, respectively.

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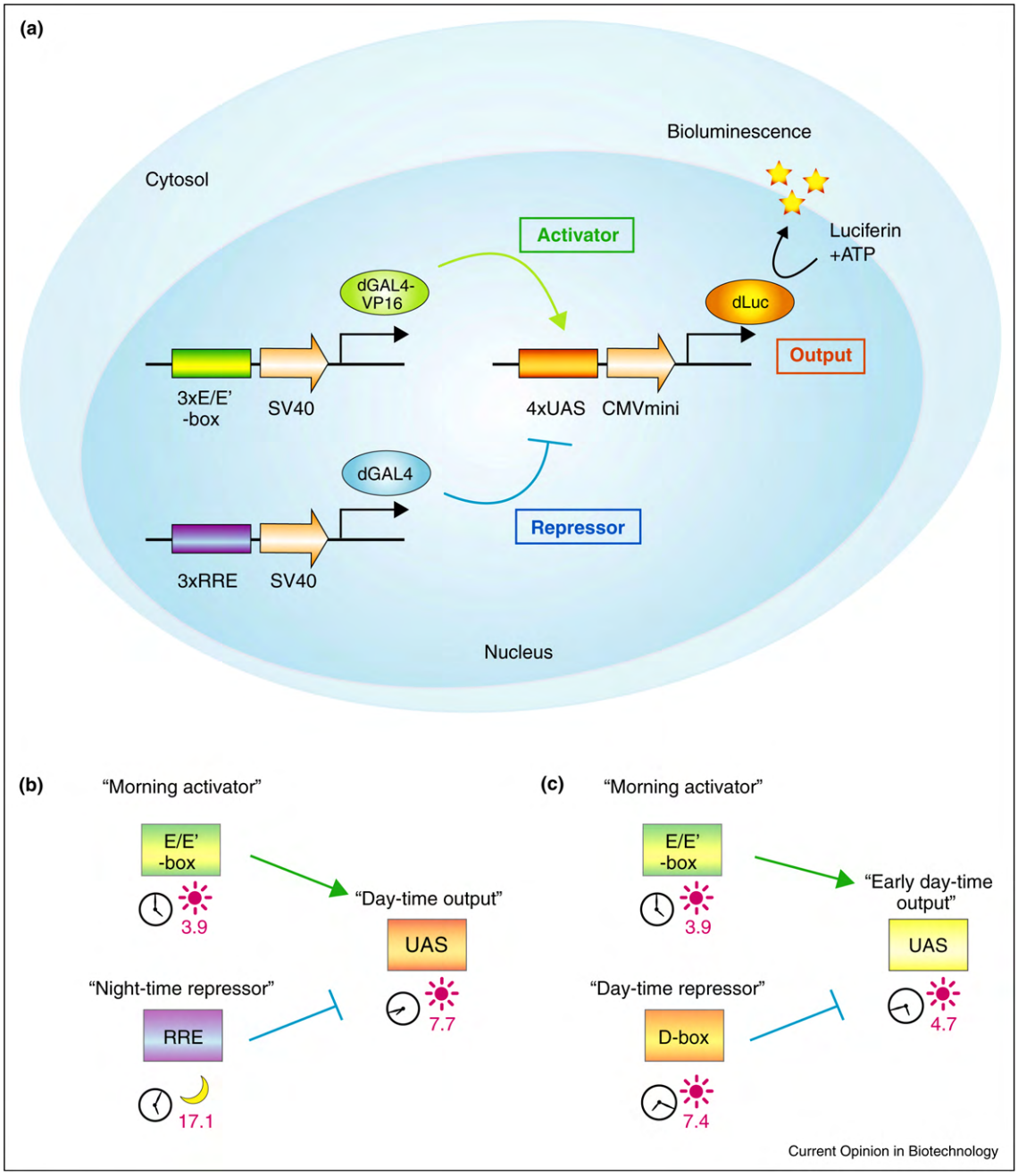
Figure 2



Synthetic clock outputs with a natural wiring mechanism. **(a)** Ukai-Tadenuma and colleagues developed an artificial *in vitro* transcriptional cycling assay system in mouse NIH3T3 cells to determine whether *cis*-elementary transcriptional regulations via the three main CCEs (the E/E′-box (morning), the D-box (day-time), and the RRE (night-time)) are sufficient to create clock outputs. This system was composed of an artificial activator (destabilized GAL4 fused to VP16 transcriptional activator; dGAL4-VP16) and an artificial repressor (destabilized GAL4; dGAL4), which were expressed under the control of either of CCEs (3 × CCE) and regulate the expression of destabilized *luciferase* (*dLuc*) reporter gene via competitive binding to its upstream activator sequences (four tandem repeats of the GAL4-binding sequence; 4 × UAS) fused with a minimal CMV promoter (CMV mini). Using the assay system, the investigators reconstructed a natural circadian output, **(b)**, day-time in this example, using a morning activator and night-time repressor as suggested from previous information. They also successfully designed an unnatural, artificial circadian output with various combinations of these CCEs with the transcriptional regulators, **(c)**, early day-time in this example, using a morning activator and day-time repressor.

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associate with the BMAL1/CLOCK heterodimers to inhibit E-box-mediated transcription [2••,3••,5•,7•]. However, the mechanisms behind the circuit's dynamic properties remain largely elusive.

Reconstruction and design of circadian transcriptional networks

Simple regulatory modules, such as positive and negative feedback loops, can generate complex dynamic behaviors, alone or when they are embedded in larger network structures. Theoretically, a specific type of gene regulatory network can display a particular dynamic behavior, such as toggle switching, logic gating, or oscillations [8]. Recent work in synthetic biology focuses on forward engineering gene regulatory networks similar to those observed in known biological networks, and proving the natural circuits' design principles by synthesis ('proof-by-synthesis'). The first step in designing a network structure with specific dynamic behavior is often *in silico* modeling; this is followed by an experimental implementation of the designed molecular network *in cellulo*.

Several studies have attempted to design and implement purely artificial networks of *de novo* wiring to generate systems exhibiting oscillatory behavior, a characteristic dynamic behavior in the circadian system, by using a repressilator transcriptional network [9•], coupled positive and negative feedback transcriptional loops [10–12,13•14•], or a transcriptional- and metabolic-integrated network loop [15]. A study by Elowitz and colleagues was one of the earliest synthetic approach in which they designed and implemented the by now 'classical' repressilator gene circuit into *Escherichia coli* [9•]. Later, more robust — and tunable — oscillation systems were achieved in mammalian cells [12] as well as in *E. coli* [11,14•].

A challenge of the reconstruction or design of a circadian oscillator with a relatively long period (nearly 24 h) and stable amplitudes has not yet been accomplished. Recently, Tigges and colleagues successfully generated an artificial transcriptional network with an oscillatory period of about 26 h, but with fragile oscillations [13•]. Another attempt is to use the natural components of the circadian clock, which was performed by Chilov and colleagues [16•]. They tried to reconstruct a feedback loop of the natural circadian oscillatory network, composed by inducibly expressed Bmal1/CROCK gene plus E/E′-box-connected Per/Cry gene and/or a reporter gene. The system performed at least a single cycle of a clock-like oscillation, but sustained oscillatory expression of the reporter gene was not observed. Thus, it appears difficult to artificially reproduce periodic dynamic behavior with a sustainability and relatively long period (nearly 24 h). An alternative approach to build a stable circadian oscillator is to utilize wiring information in a natural circadian transcriptional network [17••]. They succeeded to reconstruct, at least in part, sub-network of mammalian circadian clocks, and found that the phases of the transcriptional activator(s) and repressor(s) of the circadian clock can determine the downstream transcriptional output phase, by using an *in vitro* cycling assay system composed of an artificial activator and repressor, of which expression timings were controlled via either of CCEs, and an output reporter gene regulated by the activator and repressor (Figure 2a). The artificial transcriptional circuits successfully reproduced the natural circadian output and they generated other unnatural phases by various combinations of these CCEs with the transcriptional regulators (Figure 2b,c). However, they could not regenerate the morning phase. Taken together, the challenges of synthesizing a 'perfect' natural circadian clock remain to be solved, as we work toward the complete reconstruction of the transcriptional circuits underlying the mammalian circadian clock.

We also note that synthetic-biological approaches can be applied to other dynamic properties of circadian clocks such as circadian output's amplitude, temperature compensation and time delay. For example, artificial CCEs were synthesized followed by their implementation into cells [18•]. Among the synthetically designed CCE sequences, there were *cis*-elements with very high- or low-amplitude circadian transcriptional activity. High-amplitude oscillations required an appropriate affinity balance between the activators and the repressors. Thus, novel design principles and underlying mechanisms were discovered by the synthetic approach. In addition, temperature compensation of the molecular clock is another target of the synthetic-biological study, although it seems necessary to design temperature-insensitive enzyme [19]. Other synthetic approaches tried to create time-delay circuits with feed-forward loops or multistep reactions [20–22]. Such time-delay circuits can be an important control motif in both circadian clock and other signaling pathways in nature. Overall, these proof-by-synthesis approaches with natural circadian components serve a dual purpose; they can investigate sufficiency of identified natural components and/or their interactions, and also reveal requirement of previously unidentified components or interactions.

Design and implementation of dynamic properties of the central clock tissue

The circadian clock adjusts an organism's metabolic and physiological activities to the environmental day–night cycle of the earth. In mammals, organism-level circadian rhythmicity is critically regulated by one central pacemaker, located in the SCN of the hypothalamus [3••,4••,6••]. The central clock tissue is of particular interest, because it regulates many cyclic metabolic and physiologic processes, such as the sleep–wake cycle. Thus, reconstruction of the dynamic properties of the SCN will be among the next applications of synthetic approaches for the mammalian circadian clock.

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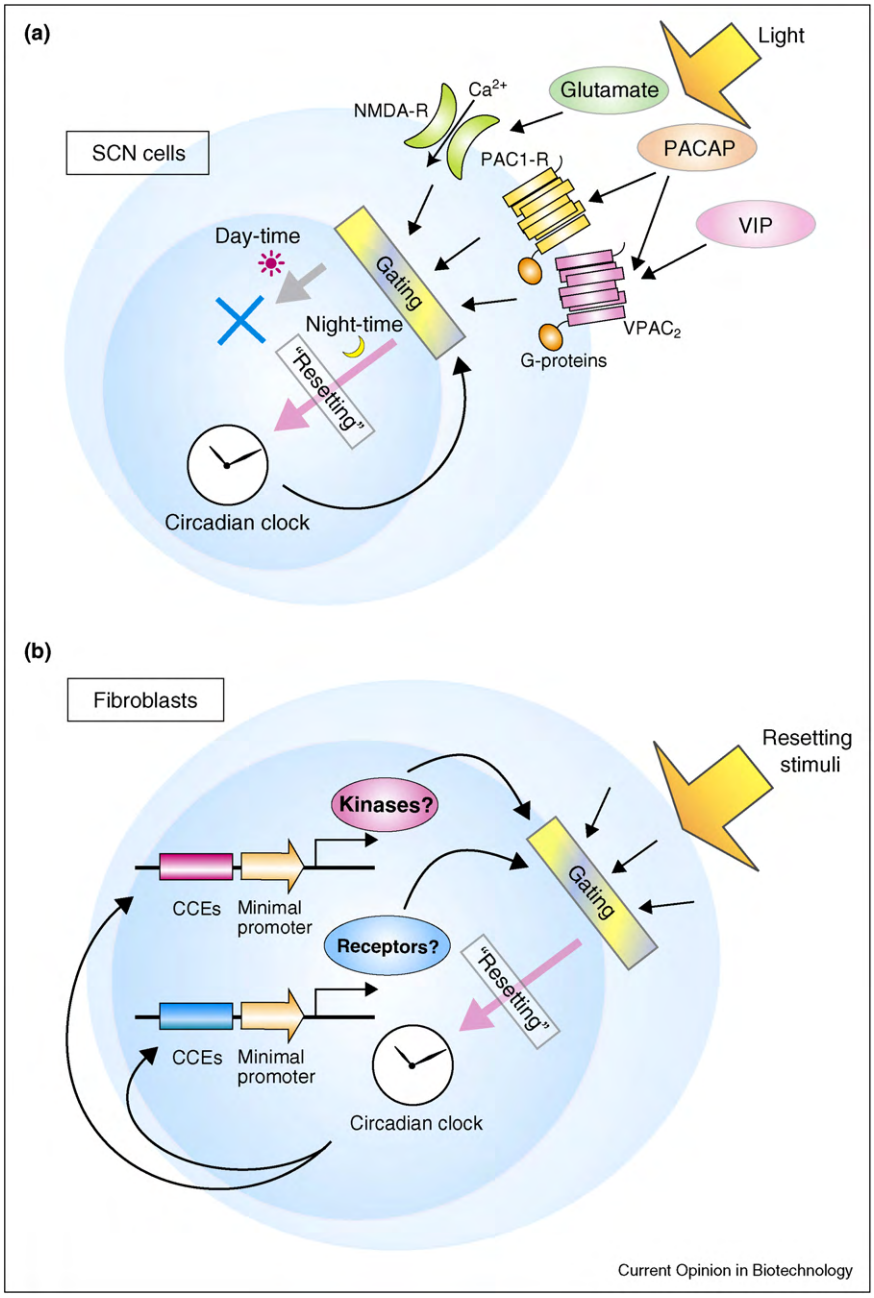
Figure 3



A synthetic approach toward creating the gating property of the SCN. **(a)** A phase-dependent sensitivity to external stimuli such as light is one of the fundamental properties of the circadian clock. Photic information from the retina is delivered through glutamate and PACAP of the RHT. VIP is also proposed as an intrinsic SCN factor mediating the photic signaling. These neurotransmitters bind to the receptors expressed at the surface of SCN cells and activate intracellular signaling pathways. The signaling pathways may be regulated by the circadian rhythm to display phase-dependent sensitivity to the extracellular signals. In fact, the photic signal transduction system selectively delivers the information of light only at night and it entrains the circadian clock. **(b)** In a synthetic approach, researchers may be able to implement a designed transcriptional circuit composed of the signaling molecules regulated by CCEs into a non-SCN cell type (e.g., fibroblast) to execute the property of the phase-dependent resetting of circadian clock by external stimuli.

One of the fundamental dynamic properties of the central circadian clock is *gating*, a phase-dependent response to external stimuli. For example, exposure to light during subjective night-time, but not during subjective day-time, can effectively entrain the central circadian clock [3••] (Figure 3a). In the photic response program, glutamate and pituitary adenylate cyclase activating peptide (PACAP) in the retinohypothalamic tract (RHT) are

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thought to be the main neurotransmitters that deliver photic information to the SCN; they convey critical information for photic entrainment [4••,5•,6••,23••]. More specifically, glutamate binds and activates the NMDA receptors of SCN neurons and it induces intracellular calcium-dependent signaling pathways, which in turn upregulate *Per1* and *Per2* in the SCN cells [3••,4••,6••,23••] (Figure 3a). PACAP binds to G-protein coupled receptors (PAC1-receptor and VPAC2) of SCN cells [23••] (Figure 3a). Intrinsic neurochemical signaling among the SCN neurons, mediated by vasoactive intestinal polypeptide (VIP) and its receptor VPAC2, has also been proposed as an important factor in the photic entrainment of the mammalian clock [23••,24] (Figure 3a). Mathematical models of photic entrainment can help predict the efficiency of multiple control targets and their combinations for circadian phase resetting, as studied by Bagheri and colleagues [25].

These extracellular stimuli activate several intracellular signaling molecules. It is not clear how the gating process is implemented in the signal transduction pathways associated with these components; however, several of these proposed factors, including *Ryr2* mRNA, phosphorylated MAPK, cGMP levels, and PGK activity, have been reported to exhibit oscillatory behaviors [3••,4••,23••,26–28], and the oscillation of these molecules along with the circadian rhythm can prompt cells to exhibit gating of the photic response. Again, synthetic cyclic transcriptional regulation of these signal transduction pathways may provide insight into the underlying molecular mechanisms for gating in the SCN neurons.

Electrophysiological oscillations are another fundamental dynamic property of the SCN. A single SCN neuron can express circadian rhythmicity in its electrophysiological activity [4••,6••,7•,23••,29]. Recent studies provide evidence that electrophysiological oscillation in harmony with the circadian rhythm does indeed regulate changes in such physiological functions as the osmosensory reaction during late sleep [30]. Both experimental analyses and mathematical modeling suggest that this rhythmicity is partly mediated by several kinds of Ca^{+} or K^{+} channels, including L-type voltage-dependent Ca^{2+} channels, fast-delayed rectifier K^{+} channels (Kv3.1b and Kv3.2), or a Ca^{2+}-activated Bk channel (Kcnma1) [23••,29,31•,32•].

Intriguingly, the electrophysiological oscillations are associated with daily expression changes of these channels, such as the fast-delayed rectifier K^{+} channels (high in day-time) and the Bk channel (high in night-time) [23••,31•,32•]. Mutations in circadian clock genes — including a *Tau* mutation in casein kinase I epsilon or a dominant negative mutation of the *Clock* gene — change the SCN's electrophysiological rhythms in mice [29]. A recent report suggested that the *Per1* gene is involved in some electrophysiological features within SCN cells [33•], further supporting the direct interactions between the clock gene network and electrophysiological components. However, the exact nature of the interactions remains elusive. Synthetic design and implementation of cyclic electrophysiological activity in non-SCN cells may help reveal the sufficient minimal components required for cyclic electrical outputs from the SCN neurons.

A third fundamental property of SCN neurons is their ability of synchronized oscillations—they form a coupled oscillator at the tissue level against inevitable noise. More specifically, the SCN consists of ~20,000 neurons and their circadian activity is unequivocally synchronized [2••,6••]. Synchronization is indispensable to the SCN function as a central pacemaker as was revealed through the study of the singularity phenomenon [34•]. Furthermore, the SCN derived from several clock gene knockout mice showed sustained circadian oscillation although these clock genes were required for the circadian oscillation at a single-cell levels, suggesting compensation of the circadian behavior by the coupling [35•]. An impairment in the intercellular communication among the SCN cells results in desynchronization and arrhythmicity [36,37].

What are the underlying mechanism(s) for synchronization? Our understanding of collective synchronization in coupled non-linear oscillators has been derived mainly by studying simplified phase models such as the Kuramoto model [38•]. More realistic subsequent theoretical studies have refined the picture [39–44,45•]. For example, mathematical modeling of the intercellular coupling of noise-resistant circadian oscillators highlighted the importance of oscillatory factor(s) that are secreted at a specific circadian time and can induce light- or dark-pulse-type phase shifts in neighboring cells [43]. More recently, Bernard and colleagues developed a realistic model that comprises a heterogenous set of damped cellular oscillators and a coupling agent. Connectivity was simulated in the three-dimensional *in vivo* SCN or two-dimensional sliced SCN with separate core and shell compartment of the tissue [45•]. This model emphasized the importance of population size, number of oscillators, and connectivity for the synchronization. Besides the SCN, to understand the basic principle of synchronized oscillatory behavior at the population level, Danino and colleagues recently developed an engineered gene network that enable synchronized oscillations in a growing population of *E. coli* [14•], and highlighted the importance of a small secretory molecule and an appropriate cell density as well. Similar principles may also establish synchronization in the SCN, given that SCN cells are packed into a small region of the hypothalamus with intercellular connectivity [23••]. Regarding specific biological mechanisms, for instance, the VIP-VPAC2 signaling pathway is thought to mediate intercellular communication among the SCN neurons [6••,37,46]. These signaling pathways thus may provide

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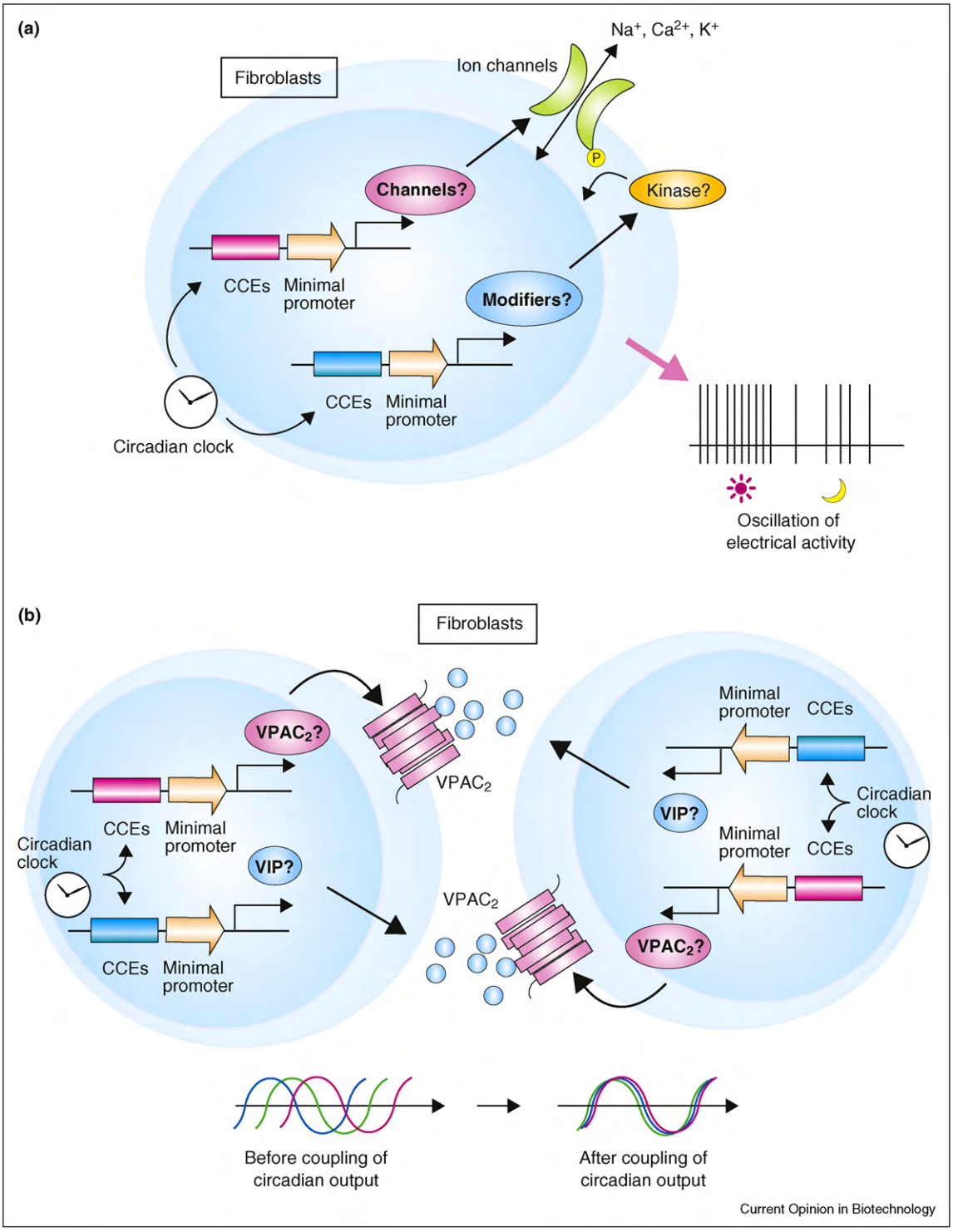
Figure 4



Synthetic approach toward designing electrophysiological oscillation and coupling in the SCN. SCN cells exhibit characteristics such as oscillatory electrophysiological activity and synchronized circadian oscillations (coupling), which are critical for SCN function as a central pacemaker. Researchers may incorporate a designed transcriptional circuit regulated by CCEs, and composed of **(a)** ion channels or their modifiers or **(b)** secretory molecules and their receptors, into a non-SCN cell type (e.g., fibroblast) to create the desired properties of an oscillated electrical activity or intercellular coupling.

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first targets for future synthetic approaches to reveal the underlying molecular mechanisms of synchronization among SCN neurons.

Taken together, we propose that a key to designing SCN function is to use and modify the mechanisms described above to generate designed outputs from the SCN (Figures 3 and 4). A possible synthetic approach is to implement these oscillatory networks in cells and reproduce the desired property. This is analogous to the study of proof-by synthesis of circadian phase where a designed transcriptional network in which the gene expression is regulated by CCEs, and thus regulated along with the circadian rhythm, is incorporated into cells [17••] (Figures 3 and 4). Observing whether the desired property is executed properly will help indicate the sufficiency of the system. These synthetic approaches at the individual network level will help to elucidate the relationship between the circadian clock and other networks in the brain that function to regulate various behavioral outputs such as the sleep–wake cycle.

Conclusions

The integration of systems and synthetic approaches will play a critical role in improving our understanding of dynamic physiological functions and help in the design of systems that show desired properties. We discussed this approach in the specific context of the circadian rhythms. Application of the synthetic approach to studies of the mammalian circadian rhythm will enhance not only this challenging research field, but for clinical medicine as well. In particular, people nowadays usually have to live in the artificial environment without the natural day–night cycle on the earth. Furthermore, recent social and medical problems such as aging of the human population, a part of which are accompanied by circadian rhythm disorders, is a key concern worldwide. Thus, the synthetic approach may become one of the best model cases that can inform biological engineering, with a potential for the analysis and treatment of many diseases.

Acknowledgements

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Papers of particular interest, published within the period of review, have been highlighted as:

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- •• of outstanding interest

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These were a series of latest reviews published in 2010 Annual Review of Physiology (the special topic section). In [2••], the authors introduced recent systems biology approach, particularly focused on the mammalian circadian clock as a model system. In [4••], the authors summarized neural connection of the SCN, a central pacemaker in the hypothalamus, and other circadian oscillators in other parts of the brain, which are seen in reward system or food-entrainable oscillator. They also introduced the functions and properties of peripheral clocks and their interactions to the SCN. In [6••], the authors particularly addressed the functions and properties of the SCN, including input and output, cell types, coupling, and robustness of SCN network. These reviews are helpful to understand the basic feature of the mammalian circadian clocks as well as to overview leading-edge studies of the research area.

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The authors developed a novel low-frequency mammalian oscillator using iterative cycles of mathematical model-guided design and experimental confirmations. The mammalian oscillator consists of an autoregulated expression unit encoding the tetracycline-dependent transactivator under control of the tetracycline-responsive promoter, and a negative feedback loop mediated by a siRNA-based silencing. The resultant system exhibited a little bit noisy circadian oscillation with periods of 26 hours, a clock-like oscillatory behavior.

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This is one of the latest synthetic study examining the potential ability of an artificial transcriptional network with a secretory factor to make synchronized oscillations at a cell population level. Using the synthetic

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genetic clocks, they suggested that the coupled oscillation arises by first, a small molecule that diffuses across the neighboring cells' membranes and mediates intercellular coupling by activating genes necessary for intracellular oscillation and second, an appropriate cell density for signal spreading throughout the population.

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In this paper, the authors tried to assembled and characterized a synthetic clock-like expression system based on key components of the mammalian circadian clock in a human non-clock cell line. The resultant system exhibited at least a single cycle of a clock-like oscillation.

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These papers are other representative examples of recent synthetic approach of mammalian circadian clocks. In [17••], the authors tried to generate natural and artificial phases of the circadian clocks using *in vitro* assay system. Although they successfully generated two of three basic circadian phases (day- and night-time), morning phase could not be generated. They also generated additional artificial circadian phases with a variety of combination between CCEs (see more detail in Figure 2). In [18•], the authors constructed a mammalian promoter/enhancer database and performed prediction and modeling of CCEs followed by *in vitro* validation of CCE sequences using comparative genomics method. Among synthetically designed CCE sequences, they found 'high-scoring' or 'low-scoring' cis-elements with very high- or low-amplitude circadian transcriptional activity, respectively. They also found that high-amplitude oscillations require appropriate affinity balance between activators and repressors.

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The authors thoroughly examined historical and the latest publications on electrophysiological property of the SCN. They discussed the mechanisms of circadian electric activity as well as the entrainment of the clock activity in the SCN.

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See annotation to [32•].

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These papers showed circadian oscillations of K+ channels in the SCN. In [31•], the authors showed that BK channels were clearly upregulated at night. They also examined BK channel-null mice (Kcnma1$^{-/-}$), in which night-specific increase in spontaneous firing rates in SCN neurons and weak circadian amplitudes in multiple behaviors were observed. They concluded that BK channels as important regulators of the firing rates. In [32•], the authors characterized Kv3.1b and Kv3.2 potassium channels in the SCN. An immunocytochemical analysis indicated that Kv3.1b and Kv3.2 channels are expressed within broad regions of the SCN and that expression of these channels is significantly higher in the day. They also showed blocking fast-delayed rectifier currents significantly reduces the firing rate of SCN neurons.

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The authors found the difference of electric activities among SCN neurons. During the day, SCN neurons expressing *Per1* sustain an electrically excited state and do not fire, whereas non-per1 neurons show the daily variation in firing activity. They tried to explain how ionic currents lead to the unusual electrophysiological behaviors of the Per1 cells using a combined experimental and theoretical approach. This paper suggested that the *Per1* gene is involved in some electrophysiological features within SCN cells, supporting the direct interactions between the clock gene network and electrophysiological components.

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The authors investigated the mechanisms of singularity behavior in circadian clocks, the loss of robust circadian rhythms following exposure to a stimulus such as a pulse of bright light. They reported that a critical light pulse drives desynchronization of individual cellular clocks which underlies singularity behavior. They further observed that the desynchronization underlies the multi-cell-level amplitude decrease in the rat suprachiasmatic nucleus, supporting the idea that the desynchronization is a plausible mechanism for the observable singularity of circadian clocks.

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They studied the effects of genetic loss of *Per1*, *Per3*, *Cry1*, or *Cry2* genes on the central and peripheral circadian clocks, of which output was detected using bioluminescence imaging to monitor *Per2* gene expression. While the dispersed neurons derived from the *Per1*$^{-/-}$ and *Cry1*$^{-/-}$SCN were arrhythmic in the population levels, SCN slice exhibited sustained oscillation of the reporter gene, suggesting the importance of intercellular coupling to the stabile and sustained circadian rhythms. They also used mathematical simulations to complement their in *vivo* observation.

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The Kuramoto model is one of the most representative, classic models of coupled phase oscillators proposed by a physicist Yoshiki Kuramoto. This model consists of a population of coupled phase oscillators, each having intrinsic non-linear oscillatory frequencies and mutual connectivity among the oscillator, and describe the corporate dynamics of the oscillator. This model is simple enough to be mathematically tractable, yet sufficiently complex to be nontrivial. Thus, the model is a fundamental synchronization model which is flexible to be adapted to many different contexts.

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In this study, the authors used a realistic model which is comprised of a heterogenous set of damped cellular oscillators and a coupling agent. The connectivity was simulated as the three-dimensional (3D) *in vivo* SCN or two-dimensional sliced SCN with separate core and shell compartment of the tissue. Using the model, they found the importance of population size, the number of oscillators and connectivity for the synchronization as well as predict that coupled cells either synchronize or lose rhythmicity, but do not run out of phase, demonstrating the codependence of rhythmicity and synchrony.

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