MODELING APPROACHES OF THE CIRCADIAN CLOCK AND LIGHT ENTRAINMENT IN ZEBRAFISH

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Abstract

A circadian clock is the daily time-keeping mechanism internal to most living entities. It allows the organism to anticipate and thus adapt to environmental fluctuations. While these rhythms can free-run in constant conditions, such as constant darkness, they are usually entrained to the local time by environmental cues, often light. Endogenous circadian oscillators can be found in many cells and peripheral organs, but in most higher organisms a central circadian pacemaker is present in the central nervous system, such as the suprachiasmatic nucleus in mammals. Interestingly, in zebrafish no central clock has been found, and instead all cells appear to be directly entrained by light, making it a unique and interesting vertebrate model. The circadian clocks of several species have been modeled, with interlinked feedback loops as a recurring network motif. While the mechanisms that give rise to stable oscillations are now better understood, the way entrainment works requires more investigation. The direct light entrainability of zebrafish cells makes them a great model to look into this question.

Key words

zebrafish, circadian clock, entrainment, light

1 Introduction - circadian clock

The circadian clock is the approximately 24-hour endogenous rhythm that keeps track of earth's rotation around its axis and its accompanying changes in light, temperature and humidity etc. This clock can free-run in constant conditions, but is usually entrained and can be phase-shifted by environmental signals. It is also temperature compensated, i.e. keeps the same period even in different temperatures. Notably, circadian clocks are present is a wide number of organisms from higher organisms to cyanobacteria. It thus appears that the daily and also seasonal anticipation of the environment and subsequent synchronization of processes has a huge effect on the organism's fitness. The correct timing of, for example, photosynthesis or social interactions is crucial, and it has been shown in several species that a functional clock increases fitness. There are some especially interesting features to consider in the context of circadian clocks: why it is not just a simple hourglass timers counting down time from dawn; the presence of interlocked positive and negative feedback loops increasing stability and tuneability; that multiple clocks may be present in one organism resulting in different peak phases.

Clocks in several species have been investigated in detail. While the exact wiring diagrams are not the same, there are features that are used over and over again. Mathematical modeling has been able to significantly shed light on how molecular oscillations may arise in different organisms. Often these self-sustained molecular oscillations are based on interlinked feedback loops, allowing tunability at the same time as robustness. These systems may appear basic in structure, but they exhibit complex emergent properties, some of which are not well understood. Research in this area receives a lot of interest, not least due to several medical interests from treating jet lag and sleep disorders to the optimal timing of drug administration.

2 Circadian clocks in different species

To understand circadian clocks, this section will provide a short description of the clocks and modeling approaches in different species. While there may have been a basic clock mechanism in a common ancestor e.g. before separation of insects and mammals, circadian clocks have probably evolved several times. The basic layout has also been extensively modified and extended over the course of evolution, e.g. through the inclusion of central pacemakers and sensitivity to new environmental cues. There is some use of orthologs between mammals, flies and maybe even fungi, however, cyanobacteria circadian proteins seem unrelated.

Several circadian proteins have certain domains which are highly conserved in a range of species, such as the DNA binding basic Helix-Loop-Helix (bHLH) domain and the protein-protein interacting PAS domain. Often there is a transcriptional activator, that is active as a heterodimer, and one or more negative elements. There may also be elements with a dual activator and inhibitor role in different loops. To entrain to light, some proteins act as a light input pathway. Input signals of this kind alter the level of one or more components of the loops in order to reset the phase of the rhythm, advancing or delaying it. Models of these translation-transcription networks are often described by equations with MichaelisMenten kinetics to model enzyme-mediated degradation of gene products, Hill functions to represent transcriptional activation and linear translation rate and the rate of protein transport in and out of the nucleus. As the exact rate constant are mostly unknown, parameter values set can be selected with optimization to reproduce experimental results, such as correct period, phase and entrainment. Also note that multiple types of posttranscriptional regulation and trafficking play a role in the clock and may thus be treated as different states in a model.

2.1 Cyanobacteria

While 30 years ago only eukaryotes were believed to have circadian clocks, we now know that cyanobacteria are one of the simplest organisms to exhibit circadian rhythms. In the cyanobacterium *Synechococcus elongatus* the three genes *kaiA*, *kaiB*, and *kaiC* are essential components of the circadian clock. While it was first assumed that a transcription and translation feedback loop was the basis of the clock, it was later found that the KaiC phosphorylation cycle, which persists even without transcription or translation, is the key process: in a test tube it was possible to reconstituted a self-sustainable temperature compensated oscillation of KaiC phosphorylation with only the three Kai proteins, KaiA, KaiB, and KaiC and ATP (Nakajima, Imai, Ito, Nishiwaki, Murayama, Iwasaki, Oyama and Kondo, 2005). Oscillations of the Kai proteins are believed to result from KaiA sequestration by KaiC hexamers and KaiBC complexes and has been modeled by differential equations stating the phosphorylation state of KaiC.

2.2 Fungi Neurospora

The fungus *Neurospora crassa* is a comprehensively studied and well understood circadian system. The output is a 22 hour rhythm in asexual spore formation in constant darkness, as well as other circadian rhythms in, for example, metabolism and stress response. The central components include the rhythmic gene frequency (*frq*) and the constitutively expressed genes white collar-1 (*wc-1*) and white collar-2 (*wc-2*), which form a heterodimeric white collar complex (WCC) via PAS domains, comprising the positive elements by activating transcription of *frq*. When FRQ accumulates, it inhibits WCC's activation of *frq* transcription, thus closing the negative feedback loop. In addition, FRQ positively regulates expression of WC-1, resulting in a positive feedback loop interlocking with the primary loop. See Figure 1. Photoentrainment occurs through the blue-light photoreceptor WC-1. Light-activated WC-1 enhances transcription of frq by making up a slower migrating WCCs complex.

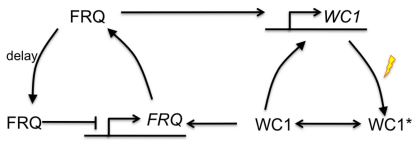

Figure 1. *Neurospora* Model Network diagram.
Interlocked feedback loop model of the *Neurospora* circadian clock (Akman et al., 2010). WC-1 is the positive element, while FRQ is the negative element. FRQ also upregulates the level of WC-1, yielding a positive interlocked feedback loop. WC1* represents light-activated WC-1. There is a delay between the translation of FRQ and conversion into its active form.

2.3 Plants

The plant circadian cock has been found to regulate many biological processes, about a third of the transcriptome of the plant model, *Arabidopsis thaliana*, and its function increases fitness and biomass. *A. thaliana* has been investigated experimentally and with mathematical modeling to suggest a model with three interlocked transcriptional-translational feedback loops. In the first negative feedback loop, two closely related, partially redundant dawn-phased MYB transcription factors, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LONG ELONGATED HYPOCOTYL (LHY), inhibit directly the expression of an evening-expressed gene, pseudo-response regulator TIMING OF CAB EXPRESSION 1 (TOC1), while TOC1 in turn upregulates the expression of CCA1 and LHY via a still unknown factor X. In the second, or morning loop, the expression of CCA1 and LHY is inhibited by morning-phased clock components, such as PRR9, PRR7 and PRR5. While single mutant phenotypes are subtle, the prr5 prr7 prr9 triple mutant is essentially arrhythmic. A further evening negative feedback is formed by an unknown component Y, that positively regulates TOC1 expression and is negatively regulated by TOC1, CCA1, and LHY. The evening-expressed GIGANTEA (GI) has been suggested to play a role here. For a diagrammatic representation see Figure 2.

How light entrainment is achieved is still unclear, but it may occur via modulation of multiple clock genes at different regulatory levels. Expression of CCA1, LHY, PRR9, and GI is induced by light and these are target genes for light resetting. Light also promotes degradation of CCA1 mRNA and increases the translation rate

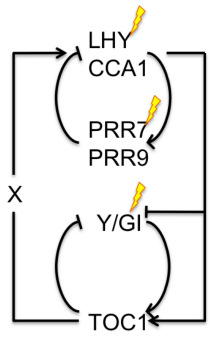

Figure 2. Plant Model Network diagram.
Three interlocked feedback loop model of the plant circadian clock as modeled by (Locke et al., 2006). CCA1/LHY inhibit TOC1 which in turn activates CCA1/LHY via X. A morning and evening loop are interlocked.

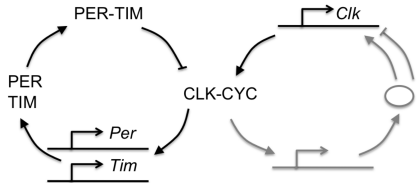

Figure 3. *Drosophila* Model Network diagram.
Simplified interlocked feedback loop model of the *Drosophila* circadian clock as modeled by (Fathallah-Shaykh et al., 2009). PER and TIM proteins accumulate and dimerize and inhibit CLK/CYC. Several other factors that make up interlocked feedback loops are summarized in grey.

of LHY mRNA, as well as regulating the stability of many clock proteins.

2.4 Drosophila

The fly *Drosophila* circadian clock proteins show some homology to mammalian ones. A group of 20-30 lateral neurons in the adult fly brain have been found to act as a pacemaker. Period (PER) and timeless (TIM) proteins accumulate and dimerize in the cytoplasm and translocate to the nucleus where they may dissociate. Here they bind the DNA-binding heterodimer CLOCK/CYCLE (CLK/CYC). As one of the targets of CLK/CYC are the per and tim genes, PER and TIM negatively regulate their own expression. Posttranslational regulation causes a temporal delays between CLK/CYC transcriptional activation and PER/TIM repression. Several other factors such as doubletime (dbt), shaggy (sgg) and vrille (vri) refine this with additional interlocked feedback loops. For the model, see Figure 3. To synchronize the internal clocks to the 24-h cycle of sunlight, Drosophila utilize the cell-autonomous blue-light photoreceptor Cytochrome (Cry). CRY interacts with TIM, promoting its degradation.

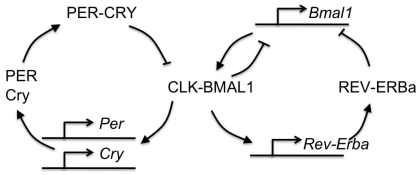

Figure 4. Mammalian Model Network diagram.
Simplified interlocked feedback loop of a more complex model of the mammalian circadian clock with 19 kinetic equations (Leloup and Goldbeter, 2003). PER and CRY proteins are phosphorylated and transported to the nucleus (not shown for simplicity), dimerize and inhibit CLK/Bmal1. REV-ERBa compromises a negative feedback loop.

2.5 Mammal

In mammals, the circadian clock again consists of several integrated feedback and feed-forward loops. While the mammalian circadian master clock is primarily located in the suprachiasmatic nucleus (SCN) in the hypothalamus and is entrained by light through the retina, different peripheral oscillators in other organs and tissues possess endogenous clocks, but these are synchronized by the SCN. Many mammalian clock genes have been identified and include bHLH-PAS transcription factors (Clock and Bmal1), period genes, cryptochrome genes and two orphan nuclear hormone receptors (Rev-Erba and Rora).

CLOCK and BMAL1 dimerize and directly, and indirectly, activate transcription of the *Per* and *Cry* genes through E-box elements. The PER and CRY proteins accumulate in the cytosol and are then translocated, following phosphorylation, into the nucleus, where they form regulatory complexes and inhibit the activity of CLOCK and BMAL1, by binding to the CLOCK-BMAL1 complex. Bmal1 expression is also subjected to negative autoregulation by BMAL1, through the product of the Rev-Erba gene. The complex between PER2 and CRY1 or CRY2 enhances Bmal1 expression in an indirect manner by binding to CLOCK-BMAL1, and thereby reducing the transcription of the Rev-Erba gene. Light can entrain circadian rhythms by inducing the expression of a Per gene though this mechanism needs further investigation. Please see the model in Figure 4.

3 Zebrafish

Zebrafish are a vertebrate model system with several similarities to mammals in the circadian clock makeup. However, no central pacemaker has been found yet and cultured fish organs and embryonic cell lines possess endogenous circadian pacemakers, that are directly light entrained. The free running period is longer than 24 hours, ca. 25hours in constant darkness, 24.4 hours in dim light (Cahill, 2002). Unlike mammalian cell cultures, which may be affected by lack of SCN input, zebrafish cell lines should give a better representation of clock functioning and especially entrainment

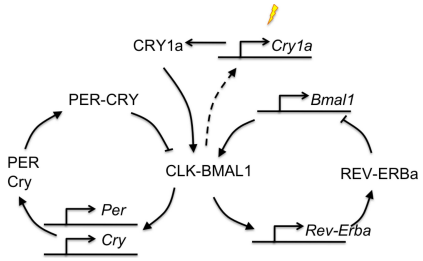

Figure 5. Zebrafish Model Network diagram.
Proposed network for zebrafish circadian clock. CLOCK and BMAL1 hetero-dimerize and activate transcription of Per and Cry genes, which in turn inhibit CLOCK/BMAL1. Cry1a is the light input to the clock. REV-ERBa might have an effect on fine tuning the clock trough an interlinked feedback loop.

in the organism as a whole. They represent a complete clock system within a single cell.
Several zebrafish clock genes have been identified and include Clock, Bmal1, three Period genes, and six cryptochrome genes. In comparison to mammals, in zebrafish there are more Cry genes, and Per2 is regulated by light as is cryptochrome 1a (Cry1a). The core clock components constitute an auto-regulatory feedback loop: CLOCK and BMAL1 hetero-dimerize and activate transcription of Period (Per) (Vallone, Gondi, Whitmore and Foulkes, 2004) and Cryptochrome (Cry) genes, which in turn inhibit CLOCK/BMAL1. In addition, it was shown that Cry1a is upregulated by light and may directly interact with specific regions of CLOCK (PAS B) and BMAL1 (bHLH, PAS B and C-terminal domains), blocking their ability to form an active dimer and initiate downstream transcriptional activation (Tamai, Young and Whitmore, 2007). Both, light intensity and the current phase of the clock have an effect on the magnitude of Cry1a induction and the resulting Per1 phase shift. Depending on the specific timing of light pulses, light can advance, delay or have no effect on the circadian rhythm, effectively resetting the clocks in asynchronous zebrafish cell cultures to a common phase. Cry1a is a strong clock repressor; remains high in the light and consequently can stop the oscillation under constant conditions. For a proposed model see Figure 5. One of the most interesting features is the direct light input to zebrafish cells. Cells left in constant darkness could be synchonised with a single 15 minute light pulse to a common phase of the circadian cycle, equivalent to the early day. This would require a high amplitude phase response curve, i.e. one capable of producing large phase shifts. Sustained light stops oscillations when the light period begins to exceed 12 h, but if light is removed the oscillator starts again from dusk. The sustained light induction of Cry1a is believed to play a critical role on this light ”stopping” response.

4 Conclusion

Circadian clocks can be exceedingly valuable asset to organisms and are believed to have evolved early in evolution. Consequently, variou s motifs and core components have been conserved across a wide range of species. However, far-reaching additions and adaptations of the basic concept can be found, especially in higher organisms, often with complex emergent properties triggered by the input of different environmental cues. The zebrafish is an attractive model in this context, as its similarities to mammalian clocks, coupled with the lack of a central pacemaker and direct light sensitivity of cultured cells, could provide great insight into the yet poorly understood processes of entrainment. Basic findings on this front can subsequently be applied to amend circadian clock models of other species.

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