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Review

How is the inner circadian clock controlled by interactive clock proteins? Structural analysis of clock proteins elucidates their physiological role

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ABSTRACT

Most internationally travelled researchers will have encountered jetlag. If not, working odd hours makes most of us feel somehow dysfunctional. How can all this be linked to circadian rhythms and circadian clocks? In this review, we define circadian clocks, their composition and underlying molecular mechanisms. We describe and discuss recent crystal structures of *Drosophila* and mammalian core clock components and the enormous impact they had on the understanding of circadian clock mechanisms. Finally, we highlight the importance of circadian clocks for the daily regulation of human/mammalian physiology and show connections to overall fitness, health and disease.

1. Introduction

1.1. Circadian clocks and physiology

Most organisms from cyanobacteria to man have developed endogenous circadian (that is about 24 h) biological rhythms [1–7] in order to adapt to the geophysical day–night cycle. Many human physiological functions and patterns of behavior underlie circadian regulation by a so-called circadian clock. Examples include sleep–wake rhythms, body temperature and blood pressure, the production of hormones or the activity of the immune system [8–11]. Additionally, a connection between circadian clocks and cell cycle regulation as well as the control of genome stability is becoming increasingly evident [12]. Disruption of circadian rhythms (e.g. due to genetic mutations, frequent jet-lag or shift-work) is linked to diverse pathogenic processes such as sleep disorders, depression, metabolic syndrome, obesity, cardiovascular diseases and even carcinogenesis [13–15] (Fig. 1A). Chronobiology arose as a scientific field to analyze these increasingly complex correlations and this subsequently led to the development of chronotherapy as a clinical application [16–18]. Furthermore, clock modulating compounds have been identified [19,20] and their potential use in the treatment of clock related and metabolic disorders is under exploration.

Circadian clocks and rhythms are synchronized to the environment by external stimuli (Zeitgeber signals), most importantly the light–dark cycle of a day's period, but also by temperature. In mammals, the central oscillator (*Pacemaker*/*Masterclock*) is located in the suprachiasmatic nucleus (SCN) of the brain's hypothalamus. Additionally, mammals possess peripheral clocks in nearly all of their organs and tissues to generate local circadian rhythms in physiology [21]. The central oscillator in the SCN is synchronized to the environmental light–dark cycle via photic cues, which are detected by the circadian photoreceptor melanopsin in the retinal ganglion cells [22] and are transmitted to the SCN via the optic nerve. In the SCN neurons, phosphorylation of the transcription factor CREB activates the transcription of *per1* and *per2* clock genes [23,24]. This in turn serves as an input to the main transcriptional feedback loop underlying the cell-autonomous molecular circadian oscillator (see below). Neuronal and humoral output signals of the SCN are perceived as input signals by peripheral body clocks to orchestrate circadian physiology and behavior in synchrony with the environment [21,15]. Different from the SCN master clock, peripheral body clocks can also be synchronized by feeding rhythms as dominant Zeitgeber signals. Irregular feeding rhythms may therefore lead to an uncoupling of peripheral clocks from the SCN master clock, which can affect fitness and even health. Notably, in our modern society, irregular food intake frequently arises from unusual rest-activity cycles due to shift work or jetlag [25,13]. As we will discuss below, the mammalian molecular clockwork provides input pathways to sense the cells metabolic, redox and energy states

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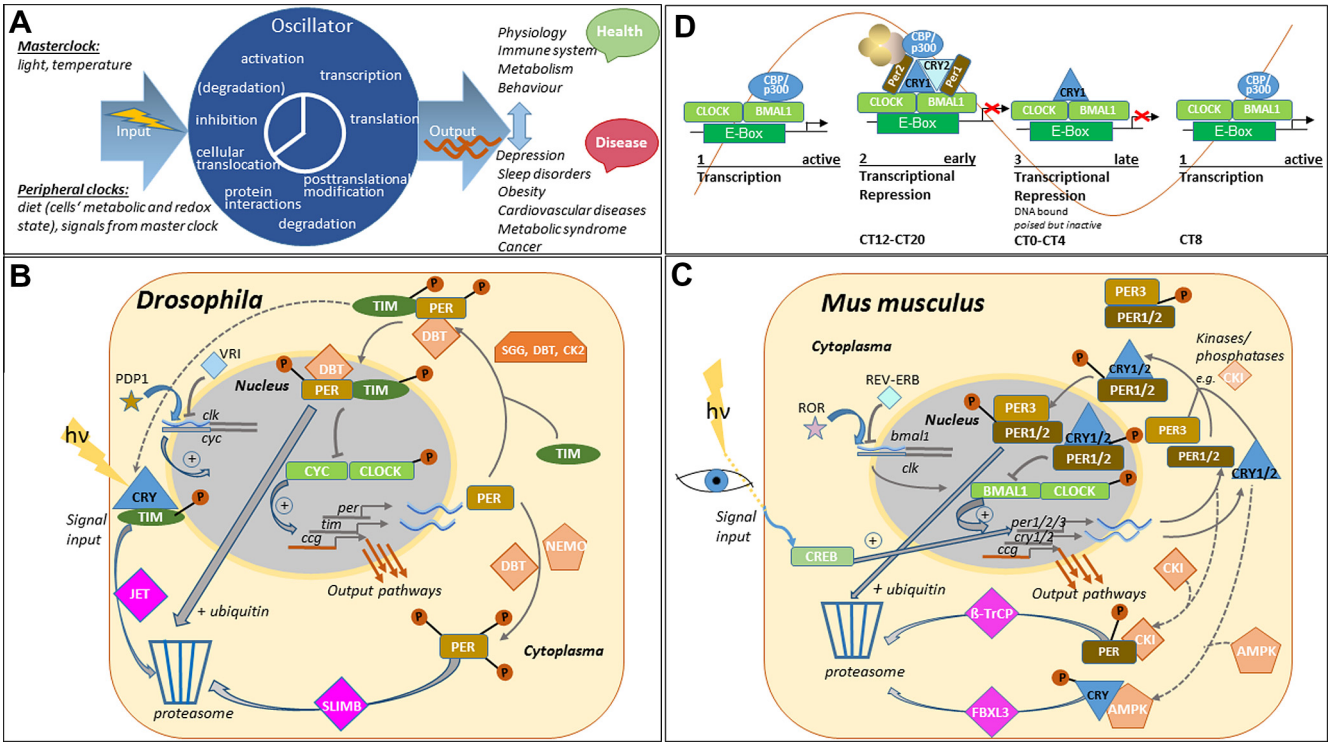

Fig. 1. Circadian physiology and molecular clocks. (A) Schematic description of the circadian oscillator and its input and output pathways. The central oscillator (master clock) time is set predominantly by light but also by temperature as environmental input signals (Zeitgeber). Oscillators in peripheral tissues (peripheral clocks) depend on metabolic steering and signals of the master clock. The input signal is integrated by the core oscillator, which is operated by cell-autonomous transcriptional and translational feedback loops. The endogenous 24 h period arises from the timely coordinated activation and repression of transcription, protein synthesis and degradation, posttranslational and posttranscriptional modifications, protein interactions and changes in cellular localization. Output signals derived from clock controlled gene activation (e.g. metabolic proteins, hormones) define circadian physiology and behavior as well as health balance. Imbalanced and dysfunctional states can lead to diseases like metabolic syndrome, obesity, diabetes, cardiovascular disease, cancer, depression and sleep disorders. (B) Molecular oscillator of *Drosophila melanogaster*. The main feedback loop including CLOCK (CLK) and CYCLE (CYC) as transcriptional activators and PERIOD (PER) and TIMELESS (TIM) as repressors is shown. In a second loop, VRI and PDP1 regulate the daily synthesis of CLOCK. Phosphorylation of PER and TIM (by kinases SGG, DBT, CK2, NEMO) as well as PER-TIM-(DBT) complex formation regulates their stability and cellular localization. The E3 ligases SLIMB and JETLAG (JET) mediate PER and TIM proteasomal degradation. TIM degradation is triggered by light-dependent interactions with the photoreceptor Cryptochrome (CRY). (C) Molecular oscillator of *Mus musculus*/mammals. The main feedback loop including CLOCK (CLK) and BMAL1 as transcriptional activators and PERIOD 1,2,3 (PER1,2,3) and Cryptochrome 1,2 (CRY1,2) as repressors is shown. In a second loop, REV-ERB and ROR regulate the daily synthesis of BMAL1. Phosphorylation of PERs and CRYs (only kinases CKI and AMPK are shown to reduce complexity) regulates their stability and cellular localization. BMAL1/CLOCK phosphorylation affects their transcriptional activity. E3 ligases TrCP1/2 and FBXL3 mediate proteasomal degradation of PER and CRY. PER1/2-CRY1/2 complexes as well as homo- and heterodimeric PER(1-3)-PER(1-3) complexes can be formed. (D) Daily transcriptional repression and activation cycles of the mammalian circadian clock. (1) In a transcriptionally active state CLOCK/BMAL1 are bound to the E-box DNA and BMAL1 recruits CBP/p300. (2) In the early transcriptional repression phase (about CT12-CT20), large complexes including CRY, PER and other factors, which affect chromatin structure and transcription termination (empty bulks), are recruited to BMAL1/CLOCK. (3) During the late transcriptional repression phase (about CT0-CT4) a repressive DNA-bound BMAL1/CLOCK/CRY complex is formed. (1) Dissociation of CRY and recruitment of CBP/p300 leads to reactivation of transcription at about CT8. CT = Circadian time; CT 0 = sunrise/light on/activity onset in diurnal organisms; CT 12 = sunset/light off. Fig. 1D was adapted from [46].

and their changes upon feeding and fasting. These input pathways are sensitive e.g. to changes in cellular levels of NAD^+ or ATP.

1.2. Molecular circadian clocks of Drosophila melanogaster and mammals

In the model organisms *D. melanogaster* and mouse, that we want to focus on in this review, the circadian oscillators have been extensively analyzed at molecular level [26,6,7]. They are operated by cell-autonomous gene-regulatory feedback loops with an endogenous free-running period of roughly 24 h. The molecular oscillators are composed of transcriptional activators and repressors. Additionally, a number of proteins (e.g. kinases, phosphatases, E3 ubiquitin ligases, (de)acetylases) post-translationally modify their target proteins and thereby periodically regulate their transcriptional activity, cellular localization, interactions, stability and cellular concentration. Physiological outputs are generated by the regulation of clock controlled genes (*ccgs*), which constitute about 10% of all genes and vary in a tissue-specific manner. In the following, key elements of the *Drosophila* and mammalian circadian oscillators will be described (Fig. 1B and C).

1.2.1. The Drosophila circadian oscillator

In the main gene regulatory feedback loop of the *Drosophila* circadian oscillator (Fig. 1B), the basic helix-loop-helix (bHLH) PAS (PERIOD; ARNT; SINGLEMINDED) transcription factors dCLOCK (dCLK) and dCYCLE (dCYC) form a heterodimer, which binds to the E-box (*enhancer box*) promoter region of the clock genes *period* (*per*, dPER) and *timeless* (*tim*, dTIM) and thereby activates their expression [26,6]. The concentration of newly synthetized dPER and dTIM proteins stays low to begin with, since protein accumulation is delayed by their proteasomal degradation. dPER degradation is initiated by phosphorylation through DOUBLETIME (DBT), a homolog of casein kinase I (CKI), which leads to the recruitment of the E3 ubiquitin ligase SLIMB. DBT phosphorylation in turn is primed by the kinase NEMO [27–29]. After sunset (around circadian time (CT) 12; see glossary for definition of CT), the cytosolic concentrations of dPER and dTIM reach their highest level. dTIM binds to the dPER-DBT complex and thereby stabilizes dPER. Phosphorylation of dTIM by SHAGGY (SGG), a homolog of glycogen synthase kinase 3 (GSK3), as well as dPER phosphorylation by casein kinase 2 (CK2) promote nuclear entry of PER-TIM-DBT complexes. In the nucleus, dTIM and dPER associate with and repress

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