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Review Article

Clockworks in the Central and Peripheral Organs: from Clock-related Genes to the Physiological and Pathological Rhythms

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Daily rhythms such as sleep-wake, feeding, and the core body temperature, persist with a period of approximately 24 hr even in the absence of environmental time cues, suggesting the existence of an endogenous time-keeping system, the circadian clock. In mammals, the circadian clock is located in the suprachiasmatic nucleus of the hypothalamus (SCN). Recently, a number of studies have revealed that circadian oscillations in the SCN are driven by the intracellular transcriptional and post-translational negative feedback loop formed by several clock-related genes such as *Period* (*Per*) and *clock* genes. Surprisingly, this feedback loop was found in many peripheral organs, indicating that the physiological and pathological rhythms in the peripheral organs were generated by the local clock in the peripheral organs, which synchronize to the central clock in the SCN. In addition, several humoral factors seem to mediate communication between the central and peripheral clocks. Furthermore, the transcription of some genes encoding the disease risk factors were found to be directly regulated by the clock genes, suggesting the possible involvement of clock genes in the onset of some diseases. In this article, we review the current view on the molecular mechanism underlying circadian oscillations in the central clock within the SCN and the local clock in the peripheral and discuss the relationship between clock genes, and physiological and pathological rhythm.

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Introduction

When stay up over the whole day, we often feel that arousal levels or drowsy state change in a 24 hr period. These daily physiological rhythms are called as the circadian (circ=about, dian=day=24hr) rhythms, since these rhythms persist with approximately 24 hrs under conditions without environmental timing cues. In addition, a lot of physiological functions such as body temperature, heart rate, energy metabolism, cell proliferation and plasma hormone levels are known to exhibit circadian rhythms in humans (Aschoff et al., 1971). Furthermore, the clinical studies revealed that the various diseases including hypertensions, cardiac diseases, strokes and asthma occur frequently at preference time of a day (Kessler, 2002).

Through the 1970's to the early 1980's, it was established that in mammals circadian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Ralph et al., 1990), based on the evidence that (1) the surgical lesion of the SCN abolished the daily rhythms (Moore and Eichler, 1972), (2) the SCN graft restored the daily rhythms in the animals which had been arrhythmic by the SCN lesion (Silver et al., 1990) and (3) the SCN *in vitro* isolated from other nucleus exhibited circadian rhythms in the firing frequency (Shibata et al., 1982) and the energy metabolism (Rosenwasser et al., 1985). It is also known that environmental timing cues entrain or reset the circadian rhythms generated within the SCN to the exact 24 hr period (Inouye and Shibata, 1994) and the strongest entrainable factor is daily light-dark cycles i.e., photic information.

On the other hand, it has been reported that some physiological functions show circadian rhythms in a SCN-independent manner. For example, the circadian

variation of the epidermal cell proliferation or intestinal enzymes activities in the intestinal tract persists in the absence of the SCN and rather strongly entrains to a circadian feeding schedule within a range of 23-30 hr (Qiu et al., 1994). The existence of these SCN-independent rhythms presumed circadian oscillators so called "local clock" outside the SCN.

In 1997, two clock-related genes in mammalians were identified; one gene named "*clock*" was cloned by the positional cloning using chemically mutated mice (*clock* mutant mice) (King et al., 1997) and another is *Period1* (*Per1*) gene which was cloned as mammalian ortholog of the fruit fly clock-related genes, Per (Tei et al., 1997). At present, a lot of studies have described the core clock mechanism involving a transcriptional and translational negative-feedback loop (King and Takahashi, 2000) in which the transcription of three *Per* genes [*Per1* (Shigeyoshi et al., 1997; Sun et al., 1997), *Per2* (Shearman et al., 1997), *Per3* (Takumi et al., 1998; Zylka et al., 1998)] are driven by the CLOCK:BMAL1 complex and negatively regulated directly by the Per proteins and the products of two *cryptochrome* genes (*Cry1* and *Cry2*) (Kume et al., 1999) (Fig. 1). Surprisingly, this feedback loop was found to function not only in the SCN but also in many peripheral organs. Therefore, one can assumed that the whole organism has both the central clock located in the SCN and the local clock in the peripheral organs. The central clock transmits the time-of-day information to the whole body and synchronizes to the environmental time cure. The local clock directly regulates the physiological functions of peripheral organs and synchronizes to the central clock in the SCN.

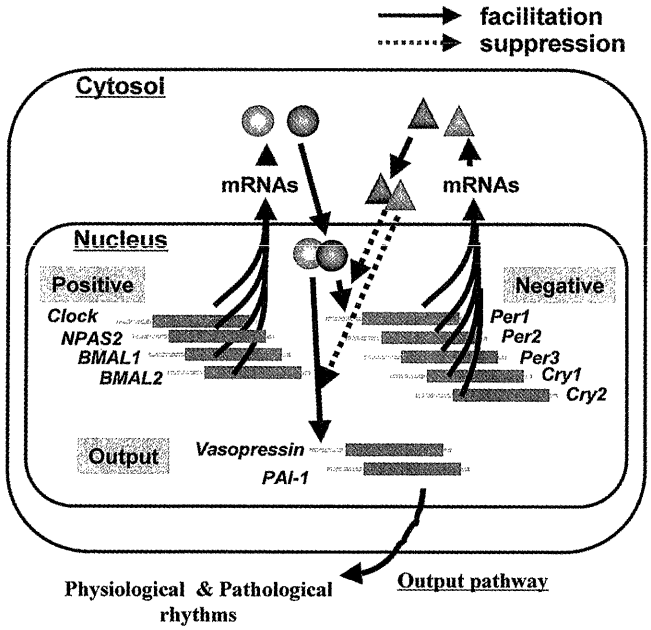

Figure 1. A Schema demonstrating the intracellular molecular mechanism, in which positive elements (*Clock, NPAS2, BMAL1, BMAL2*) and negative elements (*Per1, Per2, Per3, Cry1, Cry2*) of clock-related genes interact transcriptionally and post-translationally. Each Protein products of positive and negative elements is shown by circles and triangles, respectively. The transcription of the negative element genes are driven by the heterodimer complex of the positive element genes products and the negative elements proteins gradually accumulate in the cytosol followed by translocate into the nucleus and shut down their own transcription. Some output genes contains the consensus sequence of positive element heterodimer complex and its mRNA as well as peptide product levels was rhythmically regulated.

In this article, we first discussed the molecular mechanism underlying the circadian rhythm in the central clock in the SCN and its entrainable mechanism to the light-dark cycle. Secondary, we summarized the most recent studies on mechanisms of the local clock in the peripheral organs such as the liver and the heart. Finally, we mentioned about the feedback-regulation of the central clock by the humoral factors released from the local clock in the peripheral organs.

Molecular mechanisms underlying the circadian oscillation and the photic entrainment in the central clock within the SCN

It is becoming clear that the core clock machinery can be explained by interactions among the several clock-related genes at both the transcriptional and post-translational levels (King and Takahashi, 2000) (Fig. 1). The clock-related genes are divided into two groups; one is negative element genes, including *Per1* (Shigeyoshi et al., 1997; Sun et al., 1997), *Per2* (Shearman et al., 1997), *Per3* (Takumi et al., 1998; Zylka et al., 1998), *Cry1, Cry2* (Kume et al., 1999), *Dec1* and *Dec2* (Honma et al., 2002) and the other clock-related genes are positive element genes, such as *clock* (King et al., 1997), *Npas2* (Zhou et al., 1997), *Bmal1* (Ikeda and Nomura, 1997) and *Bmal2* (Maemura et al., 2000). The transcription of the negative element genes such as *Per* and *Cry* are driven by the heterodimer complex formed by the protein products of the positive element genes, such as CLOCK and BMAL1. As a result, Per and Cry proteins gradually accumulate in the cytosol followed by translocate into the nucleus and act as the transcriptional suppressors of the positive elements to shut down their own transcription (Fig. 1). In fact, *Per* and *Cry* mRNA levels exhibited robust circadian rhythms with a peak during daytime and a trough during the night in the SCN of mice (Shearman et al., 1997; Sun et al., 1997; Takumi et al., 1998; Tei et al., 1997), rats (Yan et al., 1999), and

hamsters (Maywood et al., 1999; Messager et al., 1999). Furthermore, gene targeting mice of *Per1*, *Per2*, *Cry1*, *Cry2*, *Clock*, *Bmal1* showed arrhythmic or abnormal rhythmicity with periods shorter or longer than 24 hr (Vitaterna et al., 1994; van der Horst et al., 1999; Bunger et al., 2000; Zheng et al., 2001). Therefore this feedback loop formed by the clock-related genes may be involved in circadian oscillations in the SCN.

Light stimulation phase-shifts locomotor activity rhythms only when the stimulation is applied during the subjective night. A transient increase in *Per1* and *Per2* mRNAs, but not *Per3* mRNA in the SCN is elicited only by photic stimulation applied in during the subjective night (Fig. 2) (Shigeyoshi et al., 1997; Zylka et al., 1998). Furthermore, central administration of an antisense oligonucleotide targeting *Per1* mRNA inhibited the light pulse-induced phase shift of locomotor activity rhythm *in vivo* as well as the glutamate-induced phase shift of neuronal firing rhythms *in vitro* (Akiyama et al., 1999). All results taken together, it is suggested that *Per1* gene is closely related with photic entrainment.

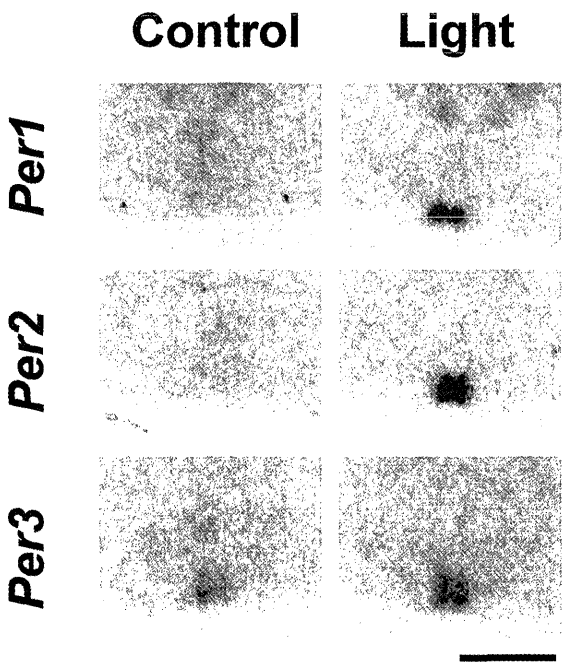

Figure 2. Representative autoradiography showing that the brief exposure to light elicits the induction of *Per1* and *Per2*, but not *Per3* mRNA in the SCN, which houses the central circadian clock. Hamster which had been kept under constant darkness conditions were exposed to light pulse (60 lux, 15 min) and were killed 90 min after. The levels of *Per1*, *Per2* and *Per3* mRNA were determined by *in situ* hybridization method using ^{33}P. Scale bar indicates 1 mm.

Photic entrainable signals are mediated via a monosynaptic afferent from the retina ganglion cells to the SCN neurons, so called "retinohypothalamic tract (RHT)". The RHT is known to possess glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) as neurotransmitters. When administrated to the SCN, both glutamate and PACAP reset the behavioral rhythms *in vivo* as well as the neuronal firing rhythm *in vitro* in a phase-dependent-manner similar to light-induced phase shifts (Akiyama et al., 1999; Harrington et al., 1999). In addition, it was reported that the antagonists of glutamate or PACAP receptors suppressed the light pulse-induced phase shifts of behavioral rhythms (Harrington et al., 1999; Moriya et al., 2000). Therefore both glutamate and PACAP may be released from the nerve terminal of the RHT upon light stimulation and cause the photic entrainment of the circadian clock in the SCN. Glutamate receptors are divided into three major subtypes; that is, N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainic acid (KA), and metabotropic receptors. NMDA receptors were proved to be principally involved in photic resetting of the circadian clock, although all three types of glutamate receptors are expressed in the SCN. We demonstrated that the activation of NMDA receptor is a critical step for photic induction of *Per1* and *Per2* transcripts in the SCN, which are linked to a photic behavioral entrainment (Moriya et al., 2000). Also, PACAP elicited an induction of *Per* expression in the SCN and its dose-dependency was well associated with that of the phase resetting of the behavioral activity rhythm (Minami et al., 2002). A receptor for PACAP, denoted as PAC(1), exists in six variant forms. Recently, we reported that the three PAC(1) variants (PAC(1)short, PAC(1)hip, and PAC(1)hop1) were densely expressed in the SCN and, interestingly, the levels of only PAC(1)short mRNA exhibited a circadian rhythm with a peak during early daytime (Shinohara et al., 2002). These results suggested that the sensitivity of the circadian clock to the phtotic entranable stimuli is directly regulated by the circadian clock, which might serve facilitate the photic entrainment.

Circadian oscillation, synchronization and output mechanism of the local clock in the peripheral organs

One of the most surprising findings in the studies on clock genes expression is that clock genes such as *Per* and *Bmal1* mRNA were abundantly expressed in the almost all peripheral organs with clear circadian rhythmicity (Zylka et al., 1998). Furthermore, Schibler and his colleagues (1998) demonstrated that the circadian expression of *Per1* and *Per2* genes was also

observed in the cultured cell lines such as rat-1 fibroblasts or H35 hepatoma cells by treatments with high concentrations of serum (Balsalobre et al., 1998). In addition, the circadian rhythms in the luminescence were observed in the acute organ culture including liver, lung, and skeletal muscle from the *Per1* promoter:luciferase transgenic rat (Yamazaki et al., 2000). These reports suggested that cells outside the SCN also possess the circadian oscillatory systems, which is called as the local clock. On the other hand, Sakamoto *et al.* (1998) demonstrated that circadian rhythms of *Per2* gene expression in the heart and retina of rats were abolished after SCN lesions, indicating circadian rhythms in *Per* expression in the peripheral organs may be primarily driven by the SCN. Therefore the local clock may be a damped oscillator and was driven by time-of-day signals from the central clock in the SCN.

What is the time-of-day signal(s) from the SCN which synchronize or entrain the local clock in the peripheral organs? Using cultured vascular smooth muscle cell model, two groups reported the candidates for the signals. Nonaka *et al.* (2001) demonstrated that the treatment of vascular smooth muscles cells with angiotensin II, a well-known vasoconstrictor, elicited a synchronous cycling of *Per2* and *Bmal1* mRNAs with a period of approximately 24 hr via angiotensin II type1 receptors. On the other hand, retinoic acid phase-shifted the circadian rhythms in the clock genes in a phase-dependent manner via a hormone-dependent complex formation of retinoic acid nuclear receptors, RARα and RXRα, with the positive element of clock-related genes such as CLOCK (McNamara et al., 2001). These studies suggested that several humoral factors cooperatively entrain the local clock in the peripheral tissues.

On the other hand, Damiola and his colleagues (2000) and Hara and his colleagues (2001) independently demonstrated that the feeding acts as the strong entrainer of the local clock in the peripheral organs. Nocturnal animals (rat or mouse) are known to eat more than 90% of total feed during the nighttime. When animals are allowed to eat only during the limited time of the daytime for a week, the phase of circadian rhythms in clock genes in peripheral organs such as liver, heart, kidney and pancreas were inverted. On the other hand, *Per* mRNA expression rhythms in the SCN were not affected by this daytime feeding schedule, suggesting that the desynchronization between the central clock in the SCN and the local clock in peripheral organs occurred under inverted feeding schedule. Because the feeding is known to change a lot of physiological parameters including the body temperature, the plasma nutrient, hormone levels and cellular energy conditions, some parameters of them may entrain the local clock. The binding activity of CLOCK and NPAS2 proteins to the promoter regions of the negative element genes appear to be highly dependent on the NAD(P)H/NAD(P)+ ratio, which is drastically changed after feeding, suggesting that the cellular energy conditions may entrain the local clock in the peripheral organs (Rutter et al., 2001). On the other hand, Terazono *et al.* (2003) reported the possibility that the sympathetic nerve systems mediate the feeding entrainment. Daily adrenaline injections to the mice during the daytime caused the expression rhythm of *Per* mRNA in the liver similar to that in the restricted feeding schedule. Furthermore, electric stimulations of sympathetic nerve fibers innervating the liver resulted in the acute induction of *Per* genes in this organ.

Many physiological and pathological rhythms seem to be under the direct or indirect control of the local clock. Vasopressin promotor contains the consensus sequence of CLOCK:BMAL1 transcription factor and its mRNA levels was rhythmically regulated by the feed back loop consisting of clock-related genes (Jin et al., 1999) (Table 1). Many peripheral organs are exposed to disease risk factors. Recently, Maemura *et al.* (2000) have shown that plasminogen activator inhibitor-1 (PAI-1) gene was a direct target of CLOCK:CLIF (alas BMAL2) heterodimer complex and its transcription showed the circadian rhythmicity in the vascular endothelial cells (Table 1). Considering that the peak time of PAI-1 activity rhythm coincide roughly that of the coagulate activity, it is suggested that the PAI-1 rhythm directly driven by the molecular clock machinery in the local clock may account for the morning onset of myocardial infarction.

At present, the exhaustive DNA array analysis re-

Table 1. Output genes in the central and local clocks, which are regulated by molecular machinery composed of several clock-related genes.

Output gene	Tissues	Transcription factors	Sequence for rhythmic expression	mRNA peak time	Functions
Vasopressin	SCN	Clock	E-box	ZT6 (mouse)	Unknown
PAI-1	Heart, brain endothelial cells, kidney	Clock, BMAL2 (CLIF)	E-box	ZT10-17 (mouse)	fibrinolysis
cholesterol 7 alpha-hydroxylase	Liver	unknown	unknown	ZT11 (mouse)	neutral bile acid synthesis
HMG-CoA reductase	Liver	unknown	unknown	ZT11 (mouse)	cholesterol biosynthesis

vealed that the number of rhythmically-regulated genes in the both liver and the heart was much more than we had prospected (8-10% of the genes expressed in ach tissue) (Storch et al., 2002). These include not only the transcriptional factors, but also genes encoding some disease risk factors such as proto-oncogenes, suggesting some diseases including cancers and ischemic disease are under the control of the circadian clock. Therefore, chronotherapy against some diseases are recommended to increase the recovery of diseases and decrease side effects.

Feedback-regulation of the central clock by the local clock

A lot of studies as described above have demonstrated that the mammalian circadian system is hierarchically organized so that the central clock located in the SCN may control the local clock in the peripheral organs in order to coordinate the physiological functions in a whole body. However, little is known about the feedback regulation of the SCN central clock by the local clock in the peripherals contrariwise. For example, glucocorticoid with a high amplitude circadian rhythm failed to affect the phase of the central clock in the SCN (Balsalobre et al., 2000). Also, clock gene expression rhythms in the SCN was not changed by the restricted feeding schedule during daytime in the nocturnal mice, although the restricted feeding schedule drastically reset the local clock in the peripheral organs (Damiola et al., 2000; Hara et al., 2001). Recently, we have suggested that estrogen is a candidate for peripheral signal which could regulate the central clock in the SCN since we observed that 17-estradiol (E2) treatment extremely increased the mRNA levels of *Cry2* in the SCN of the ovariectomized rats (Nakamura et al., 2001). After E2-treatment of ovariectomaized rats, there were also significant increases in the expression of connexin-36 mRNA, a gap junction gene which is known to mediate the interneuronal communication in the SCN central clock (Shinohara et al., 2001).

In the experimental animals, estrogen modifies the circadian characteristics of running rhythms in female hamsters and rats (Morin et al., 1977; Morin, 1980). Both entrained and free-running animals in a estrous cycle show a phase advance of locomotor activity rhythms on estrous days during which blood levels of estrogen are high (Morin et al., 1977). This change in the activity rhythm throughout the estrous cycle may be caused by a direct effect of estrogen on the circadian clock, since the period of activity rhythm is shortened when estrogen is implanted in ovariectomized (OVX) rats (Albers, 1981) and hamsters (Morin et al., 1977). Together with our current studies, these behavioral studies suggest that estrogen a signal from the local clock affects the expression of the clock genes in the SCN central clock.

Furthermore, in humans, we recently found a sighted woman with premenstrual syndrome who showed menstrual changes in circadian rhythms (Shinohara et al., 2000) (Fig. 3). She showed alternative phase shifts in the sleep rhythm in the menstrual cycle: progressive phase advances in the follicular phase and phase delays in the luteal phase, suggesting that her circadian rhythms in sleep are under the control of ovarian steroid hormones. Thus, the feedback regulation of the SCN central clock by the peripheral clock is also suggested in humans.

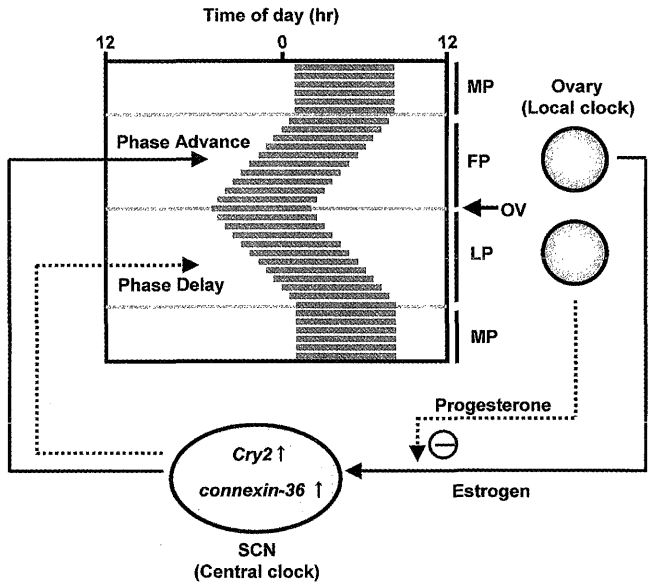

Figure 3. The feedback regulation of the central clock in the SCN by ovarian steroid hormone such as estrogen and progesterone and alternative phase shifts in the sleep rhythm in the menstrual cycle in human women. The typical sleep periods were shown by gray bars and thick and doted arrows indicate the cascade during follicular phase and luteal phase, respectively. During follicular phase, estrogen released from ovary increases both Cry2 mRNA and connexin-36 mRNA followed by advances the phase of the central clock in the SCN, leading to the continuous advancing of sleep phase. In contrast, progesterone released during luteal phase delays the sleeping phase by preventing the estrogen's actions to increase Cry2 and connexin-36 mRNA. FP: follicular phase, LP: luteal phase, MP: menstrual phase, OV: ovulation.

Discussion

In past 6 years, molecular mystery of circadian clock in the central and the local clock of mammals became clear. First, a number of current studies have given a

picture of intracellular clockwork in which the two types of clock-related genes interact transcriptionally and post-translationally. Secondly, hierarchy of the mammalian circadian clock system is understood: the central clock located in the SCN controls the local clock in the peripherals in order to coordinate the physiological functions in a whole body. Finally, the molecular mechanism underlying that some physiological and pathological events occur frequency during limited time-of-day was revealed. However, it should be noted that our picture of the mammalian circadian system is yet incomplete and a lot of unidentified factors involving the core clock machinery, entrainable signals and output signals still remained to be clarified. Also, we should address the possibility that clock genes plays a role in other functions other than circadian clocks, because *Per2* deletion mutant mice were recently proved to be cancer prone (Fu et al., 2002). There should be the connection between cell cycles and the circadian system, which has been inherited in organisms on the earth for 3.5 billion years.

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