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Molecular Mechanism of Mammalian Circadian Clock

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Circadian rhythms in behaviors and physiological phenomena of plants and animals have long been well known, but the frameworks of the molecular mechanism of circadian clocks have become clearer only within the last decade. A transcription-translation feedback loop has been shown to be an essential component of the clock, and this mechanism seems to be conserved over a wide range of species. The transcriptional activation by a Clock:Bmal1 heterodimer and the inhibition by Cryptochrome and Period are believed to provide the framework of the feedback loop in mammals. Post-translational modifications such as phosphorylation, nuclear entry and degradation have also been demonstrated to be necessary for the oscillation. Complex auxiliary loops have also been found, and these are thought to contribute to the stabilization of the feedback loop. The molecular mechanisms by which the circadian clock is adjusted to external conditions such as daily light-dark cycles, and by which the oscillation of the feedback loop is transferred to the peripheral organs are also discussed.

Key words: circadian rhythm, suprachiasmatic nucleus, transcriptional feedback loop.

Living things on the Earth exhibit rhythms with a period of about 24 h in their phenomena of life. These daily rhythms, called as circadian rhythms, are known to be self-sustained and synchronized (entrained) by environmental time cues such as a light-dark cycle with a period of about 24 h. In mammals, the circadian clock is present in the hypothalamic suprachiasmatic nucleus (SCN). The destruction of the SCN eliminated circadian rhythms of behaviors, hormonal levels and enzyme activities (*1*, *2*), and fetal SCN-transplantation restored circadian rhythms with circadian periods of the transplanted SCN in mammals whose SCN was bilaterally lesioned (*3*). Cultured SCN slices and isolated SCN neurons retained circadian oscillations in their electrophysiological and neuroendocrinological activities for more than a month (*4–6*). These data suggest that the machinery of the circadian clock resides in each SCN neuron. Peripheral tissues, such as the retina, pineal body, some brain regions other than the SCN, liver and kidney, and cultured cells including Rat-1 fibroblasts and NIH3T3 cells can also show damping circadian rhythms with a similar mechanism to that in the SCN (*7–10*). Recently, the group of Joseph Takahashi reported that the expression of the *per2* gene showed circadian oscillation for more than one month in peripheral tissue cultures [First World Congress of Chronobiology held in Sapporo, September 9–12, 2003]. Therefore, the peripheral clock mechanism might be maintained under a certain culture conditions.

Molecular mechanisms of the SCN and peripheral clocks seems to be different, because deuterium oxide (D_2O) given as drinking water caused the SCN clock but not peripheral ones to free-run under light-dark (LD) conditions (*11*). Furthermore, it was shown that glucocorticoid hormones could shift circadian rhythms of peripheral tissues but not the clock in the SCN (*12*). The difference between clocks in the SCN and peripheral tissues remains to be analyzed. The details of circadian rhythms in the peripheral tissues and cultured cells are described in another issue.

In this review, the molecular mechanism for generating the circadian rhythm is described in association with our recent findings on the mechanism in mammals.

The outline of the circadian clock: The transcriptional positive-negative feedback loop

The molecular mechanism of the circadian rhythm in cyanobacteria, fungi, insects, and mammals has become clearer over the past decade. Interestingly, these different organisms share a common mechanism. That is, the transcriptional positive-negative feedback loop (*13–16*).

A diagram of the feedback loop is shown in Fig. 1. Complexes of two positive elements, which are transcription factors, activate transcriptions of negative element genes. The positive elements are White Collar-1 and 2 (WC-1 and 2) in *Neurospora crassa*, dClock and Cycle in *Drosophila melanogaster*, and Clock and Bmal1 in mammals. These transcriptional factors possess basic helix-loop-helix (bHLH) and PAS (Per-Arnt-Sim) domains, form heterodimers (WC-1:WC-2, dClock:Cycle and Clock:Bmal1) and activate transcription of clock genes *via* E-box elements in the promoter regions of clock genes. The negative elements are Frequency (Frq) in *Neurospora*, Period (Per) and Timeless (Tim) in *Drosophila*, and three Periods (Per1-3) and two Cryptochromes (Cry1, 2) in mammals. The negative elements suppress transcriptional activities of the positive elements and thus decrease the synthesis of the corresponding gene products. In contrast to the positive elements, the negative

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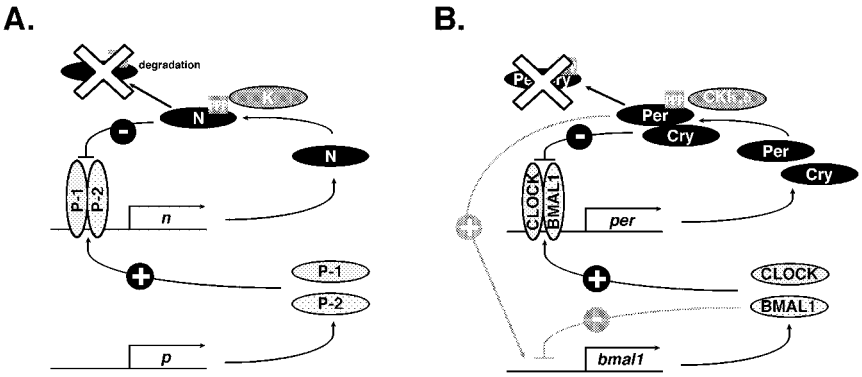

Fig. 1. **Schematic representations of the transcriptional feedback loop.** A: Common transcriptional feedback loop mechanism generating the circadian oscillations. The positive elements (P-1 and P-2) form a heterodimer and activate the transcription of the negative element (N). The translated negative element protein inhibits its own transcription by inhibition the transcriptional activation of P-1:P-2 complex. The negative element is phosphorylated by the kinase (K), and this phosphorylation may be related to the turnover of the negative element. B: The transcriptional feedback loop of mammalian circadian clock. Clock and Bmal1 are positive elements, and three Per (Per1-3) and two Cry (Cry1, 2) proteins work as negative elements. The gray arrows indicate the auxiliary loops. For simplicity, the auxiliary loop composed by REV-ERBα or DEC1, 2 are omitted.

elements do not share homologous domain structures. Despite the variability of their structures, however, they do share two common characters in their dynamics. First, their transcripts and proteins exhibit robust circadian oscillations in their expressions. The positive elements show relatively weak fluctuation or no rhythm as opposed to the negative elements in their expressions of transcripts and gene products. Second, some negative elements, Frq and Per, show a marked electrophoretic mobility shift upon phosphorylation in a time-dependent manner. The phosphorylation of the negative elements is thought to be related to their turnover, and thus with the enhancement of oscillations observed in the negative elements. Kinases responsible for the phosphorylation of the negative elements are reported to be calcium/calmodulin dependent kinase (CaMK), casein kinase I (CKI) and casein kinase II (CKII) in *Neurospora* (*17*, *18*), Doubletime (Dbt), Shaggy and CKII in *Drosophila* (*19–21*), and CKIε and CKIδ in mammals (*22*, *23*). Dbt is a homologue of mammalian CKIε and Shaggy is a homologue of mammalian glucose synthase kinase 3β (GSK3β).

Besides the mechanism described above, there appear to be auxiliary loops to secure the oscillation. In *Drosophila*, dClock negatively regulates its own transcription, and Vrille, a transcription factor, acts as a negative regulator of *per* and *tim* (*24*). In mammals, the auxiliary loops become more complex. Bmal1 also seems to act as a negative regulator of itself (*25*). The negative elements Per2 and Cry enhance the transcription of Bmal1 (*25*). REV-ERBα, which is a clock-controlled molecule and a known nuclear orphan receptor, suppresses the expression of Clock and Bmal1 (*26*, *27*). DEC1 and DEC2, which are bHLH transcription factors, are regulated by the circadian clock in their expression and act as inhibitors of the transcription induced by the Clock:Bmal1 complex (*28*). These auxiliary loops may prevent the damping of the feedback loop responsible for circadian rhythm.

The molecules that constitute the circadian clock in mammals and *Drosophila* are compared in Fig. 2.

Positive elements: Clock and Bmal1

In mammals, both Clock and Bmal1 are transcription factors possessing bHLH and PAS domains (*29*, *30*). In addition, a glutamine-rich region exists at the carboxyl terminal of Clock protein. Clock and Bmal1 form a heterodimer, which binds to E-box enhancer element (CACGTG) in the promoter regions of the *per* and *cry* genes and activates their transcriptions (*31*). *Clock*-mutant mouse has a point mutation at exon19 of the *clock* gene, synthesizes the mutant Clock protein (Clock-Δ19) lacks transcriptional activity, exhibits abnormally long periods (approximately 28 h), and loses rhythmicity in its behavior under constant dark (DD) conditions (*31*, *32*). Bmal1-knockout mouse was also reported to become arrhythmic under DD conditions (*33*) These findings suggest that Clock and Bmal1 are essential components of the circadian clock mechanism that function via their transcriptional activity. E-box is located in the promoter region of all *per* genes, and the Clock:Bmal1 heterodimer activates transcription of these genes *in vitro* (*34*). More-

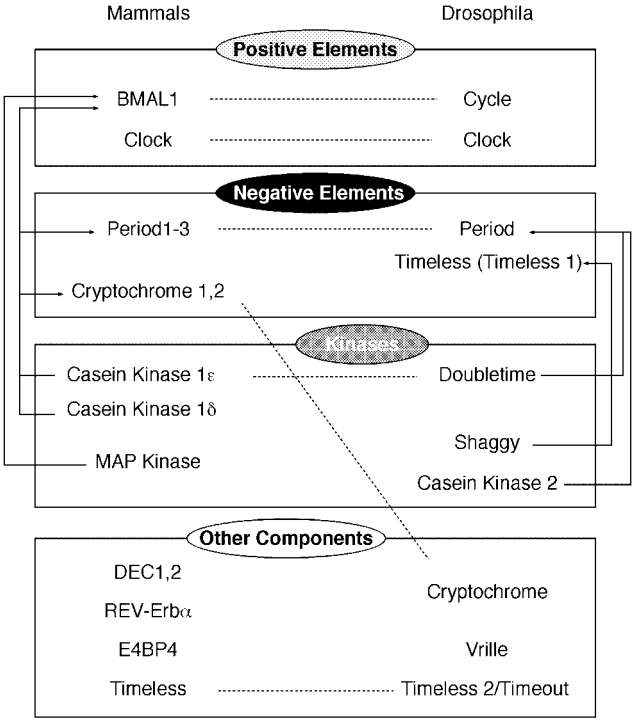

Fig. 2. **Comparison of clock-related molecules in mammals and *Drosophila*.** The orthologues are connected by broken lines. The arrows indicate the kinases and their substrates.

over, decreases in mRNA levels of *per* and *cry* in *clock*-mutant and *bmal*-knockout mice suggest that the heterodimer of Clock:Bmal1 activates the transcription of the negative elements, *per* and *cry*, *in vivo* (*33*, *35*).

Recent reports showed that SCN neurons or SCN slices of homozygous *clock*-mutant mice exhibited robust rhythms with a circadian period of approximately 28 h in their electrical activities (*36*). It is interesting that the circadian oscillation can be generated without the transcriptional activity of Clock in SCN neurons. One hypothesis is that another transcription factor compensates for the lost function of Clock. NPAS2, a family member of Clock, was reported to be able to form a heterodimer with Bmal1 and activate transcription via the E-box, like Clock (*37*). Although NPAS2 is not expressed in the SCN, another Clock family member might be expressed.

Clock does not show circadian fluctuations in its transcript and protein levels in the SCN or liver (*38*). Although Bmal1 exhibits circadian rhythms with its peak at midnight in the SCN (*39*, *40*), the amplitude of the oscillation is much lower than those of inhibitory elements Per and Cry. Moreover, it was reported that the Clock:Bmal1 heterodimer bound to the E-box throughout the day in the liver (*38*). These data raise a possibility that the positive elements have only 'passive' roles in generating the oscillations. In contrast, some data suggesting the dynamic roles of these positive elements were also reported. Bmal1 is quickly degradated by the light stimulation in the SCN (*40*). Bmal1 and Clock are phosphorylated *in vivo*, and CKIε and MAP kinase phosphorylate Bmal1 *in vitro* (*41*, *42*). Moreover, the formation of the Clock:Bmal1 heterodimer is supposed to be controlled by the redox state (*43*). Recently, it was reported that the subcellular localization of Clock in the SCN is also regulated in time-dependent manner, and that BMAL1 might be important in this phenomenon (*44*). Thus, further analysis about the roles of these positive elements is necessary.

Negative elements: Per and Cry

Mammals have two *cry* genes, *cry1* and *cry2*. Cry is a family including the blue-light photoreceptor protein in plants and (6-4) photolyase, and all members possess FAD-binding domain (*45*). However, neither Cry1 nor Cry2 has the ability to repair the DNA damage or a response to light. The mammalian *per* gene, which is homologue of *Drosophila per*, has three family members, *per1*, *per2*, and *per3* (*46–50*), and the Per proteins have PAS domains. These Per and Cry proteins exhibit robust circadian oscillations in their mRNA and protein levels, with a peak in the middle of the circadian subjective light period, and they can inhibit the transcription of their own genes by the Clock:Bmal1 heterodimer *in vitro* (*35*). Thus they are thought to be negative elements in the feedback loop of the circadian oscillation.

The $cry1^{-/-}$, $cry2^{-/-}$ double-knockout mouse was found to lose the circadian rhythmicity under DD conditions (*51*, *52*), and the transcriptional levels of *per1* and *per2* remained relatively high in the SCN of the mutant mice (*53*). These results indicate that Cry1 and Cry2 are essential for the circadian clock mechanism as inhibitory elements. The two single-knockout mice, $cry1^{-/-}$ and $cry2^{-/-}$ mice, were found to exhibit circadian rhythms in their locomotive behaviors with different circadian periods ($cry1^{-/-}$ mice, 22.5 h; $cry2^{-/-}$ mice, 24.5 h), and thus Cry1 and Cry2 seem to have different roles in the rhythm formation, although it is possible that they compensate for each other. Cry1 and Cry2 are each also known to inhibit the transcriptional activity of the Clock:Bmal1 heterodimer, but the molecular mechanism of the inhibition remains unknown (*35*). The possibility that mCry inhibits Clock:Bmal1 by controlling the redox state with its FAD is unlikely, because a mutation in the FAD binding domain of mCry does not affect its inhibitory function (*54*).

Although Per proteins are homologues of *Drosophila* Per, reports on transgenic mice of *per* suggest that functions of mammalian Per and *Drosophila* Per might be different. That is, transgenic mice of *per2* ($Per2^{Brdm}$ and $Per2^{ldc}$) were reported to lose the circadian rhythmicity in their locomotive activities under DD conditions, and transcripts of *per1*, *cry1*, and *bmal1* did not exhibit circadian fluctuations in the SCN of these transgenic mice (*55*, *56*). These data indicate that Per2 is an essential component of the circadian clock mechanism. Although Per2 is suggested to be a negative element in the circadian feedback loop, it seems not to inhibit the transcription due to the Clock:Bmal1 heterodimer, because the transcription levels of *per1* and *cry1* were reported to be lower in the Per2 mutant. One of the proposed roles of Per2 is the stabilization of Cry proteins. The formation of the Per2:Cry complex was reported to prevent the ubiquitination of these proteins (*57*). In addition, the protein level of Per2 in the $cry1^{-/-}$ $cry2^{-/-}$ double-knockout mouse is lower, even though its mRNA level remains relatively high (*58*). Another possibility is that Per2 is related to the transcriptional activation of *bmal1* (*25*).

Transgenic mice of *per1* ($Per1^{Brdm}$ and $Per1^{ldc}$) exhibited different phenotypes. $Per1^{ldc}$ gradually lost the circadian rhythmicity under DD conditions, while $Per1^{Brdm}$ did not (*55*, *56*). However, both transgenic mice retained the normal oscillation in mRNA of *per2*, *cry*,*1* and *bmal1* in the SCN. The circadian rhythm in locomotive activity gradually disappeared in *per2* transgenic mice under DD conditions, and it was lost immediately after transferring the double transgenic mice of *per1* and *per2* to DD conditions. Taken together, these findings suggest that Per1 has an important, but not essential role in the circadian clock mechanism. The transgenic mice of *per3* kept the circadian rhythmicity and the double transgenic mice of *per2* and *per3* showed a similar phenotype to *per2* transgenic mice (*55*, *59*). These data suggest that Per3 has only an auxiliary role in the circadian clock mechanism.

How Per and Cry share roles in the transcriptional feedback loop remains unclear. An immunoprecipitation study revealed that Clock, Bmal1, Per1, Per2, Cry1, Cry2, and CK1ε form complexes (*38*), but it is not known whether Per1, Per2, Cry1, and Cry2 are equally involved in the complex formation. Recently, it was shown that the double transgenic mice of *per2* and *cry2* retained the wild-type circadian rhythmicity in their locomotive activity and the transcriptional level in the SCN, but the double transgenic mice of *per2* and *cry1* did not (*60*). This suggests that the negative elements contribute unequally to the feedback loop, but further analysis is required to fully understand the mechanism.

Kinases: CK1ε, CK1δ, and MAPK

Dbt is a *Drosophila* homologue of mammalian CKIε, and is supposed to be implicated in mechanisms of the phosphorylation and degradation of Per proteins (*23*). Mutations of *dbt* gene resulted in various abnormalities in the circadian behavior. Thus, Dbt is suggested to be essential in the circadian clock mechanism in *Drosophila*. In mammals, CK1ε is also thought to be involved in the generation and synchronization of circadian rhythm. *Tau* mutant of golden hamster, which exhibits a shorter free-running circadian periods of about 22 h in heterozygotes and 20 h in homozygotes in their locomotive activity, has a sense mutation in *ck1ε* gene, and this mutation resulted in the diminution of the kinase activity (*61*). Moreover, a sense mutation at the putative CK1ε-phosphorylation site in human Per2 was shown to cause shortening of the circadian period of the sleep-wake cycles (familial advanced sleep phase syndrome; FASPS) (*62*). In COS-7 cells, CK1ε was shown to bind and phosphorylate all three Per proteins *in vitro* and to cause nuclear translocation of Per1 and Per3, but not Per2 in a phosphorylation-dependent manner (*22*). Interestingly, CK1ε was also reported to cause the nuclear export of Per1 (*63*). Another report suggested that CK1ε and CK1δ were related with the ubiquitination of Per proteins (*64*). These multiple roles of CKI seem to be caused by the phosphorylation of different serine/threonine residues, and multiple putative phosphorylation sites for the nuclear entry of Per1 by CK1ε were found in our laboratory (Takano, A. *et al*. unpublished observation). CK1ε is also reported to phosphorylate Bmal1 or Cry proteins (*41*), but the physiological significance of these reactions remains unclear.

Differences in roles of CK1ε and CK1δ in the mechanism of circadian oscillation are not clear. Since Per1 exhibits the electrophoretic mobility shift normally in *tau* mutant hamster (*38*), it is supposed that CK1δ can partly compensate for the role of CK1ε, but further analysis is necessary.

MAP kinase is also suggested to be involved in the circadian clock mechanism. Activation of MAP kinase in the SCN happened time-dependently and was induced by a light pulse in the subjective night (*65*). MAP kinase was reported to phosphorylate Bmal1 (*42*), but its function in the circadian clock mechanism is not well understood. SCOP (SCN circadian oscillatory protein), which was cloned in our laboratory as a novel clock-related protein (*66*), binds to K-Ras localized in the raft fraction of neurons through its leucine-rich repeats and inhibits the activation of the Ras-MAP kinase signaling pathway (*67*). In fact, the circadian expression pattern of SCOP was in a counter-phase of that of the Erk-phosphorylation in the SCN, that is, SCOP shows a circadian expression in the SCN with its peak in the middle of the subjective night and its trough in the subjective day (*65*, *66*). These reciprocal changes suggest that SCOP is implicated in the mechanism of the circadian clock in the SCN through inhibiting the MAP kinase activation. This must be clarified in future.

Because multiple kinases are involved in the clock mechanisms in fungi and insects, it is supposed that other kinases are also involved in the mammalian clock mechanism. One such putative kinases may be rhythmic kinase, which exhibits circadian fluctuation in its activity (*68*). The roles of kinases such as CKII or GSK3β should be also investigated.

Input pathway: How is the feedback loop adjusted?

One of the features of the circadian clock is its resetting by environmental time cues such as light. The photoreceptive pathway of the circadian clock is different between species. In *Drosophila*, Cry is a photoreceptive molecule for the circadian clock, and the retina is not necessary for the synchronization of the clock (*69*). Cry causes the degradation of Tim. The diminution of Tim leads to degradation of Per, and these degradation steps are considered to cause the phase resetting. In mammals, an environmental light signal is received by the retina and transmitted to the SCN via the retino-hypothalamic tract (RHT), and Cry is not involved in the photoreceptive mechanism (*70*). Because the input pathway of the light signal to the circadian clock is described in detail in another issue, it will be dealt with only in outline here. Light signals are supposed to be received by the retinal ganglion cells expressing melanopsin (Opn4) as well as rods and cones (*71*, *72*). The light signal is transmitted to the SCN *via* the RHT. Transmitters involved in this signal transduction pathway are glutamate, substance P and PACAP (pituitary adenylate cyclase activating polypeptide) (*70*). Glutamate is a major neurotransmitter *via* the NMDA receptors. Although the roles of PACAP in the light-induced phase shifts remain unclear, recent studies using knockout mice gradually revealed the functions of PACAP. PACAP-specific (PAC_1) receptor-deficient mice exhibit a larger phase delay by light pulse at early subjective night and a phase delay, not a phase advance, by photic stimulation at late subjective night (*73*). In PACAP knockout ($PACAP^{-/-}$) mice, the phase advance by the illumination at late subjective night was significantly attenuated, but this was not the case in light-induced phase delay at early subjective night (*74*). These reports suggest different roles of PACAP for the phase delay and advance. Evidence obtained in our laboratory suggests that tyrosine-phosphorylation of BIT (brain immunoglobulin-like molecule with tyrosine based activation motif, also called SHPS-1, SIRP-α, or p84) protein is involved in the photic entrainment of the circadian clock in the SCN (*75*). In fact, the tyrosine-phosphorylation of BIT in the SCN evoked by a monoclonal antibody against the extracellular domain of BIT caused phase shifts without photic stimulation in manners similar to responses after light exposure (*76*). In this case, an NMDA receptor antagonist, MK801, inhibited the phase advance but not the phase delay caused by the BIT antibody. These facts indicate that different mechanisms are implicated in the phase delay and advance of the circadian clock in the SCN. Glutamate also causes the activation of MAPK, CaMKII, and phosphorylation of CREB (cAMP response element binding protein) in the SCN (*65*, *77*, *78*).

One of the unsolved questions about the input pathway in the SCN is the identity of the target point of the light signal in the transcriptional feedback loop. Per1 and Per2 were considered as candidates, because *per1* and *per2* gene transcripts in the SCN are dramatically induced by the light stimulation in the subjective night (*79*). Although this hypothesis was confirmed by the use of

transgenic mice of *per1* and *per2* (*80*), light-induced *per1* and *per2* mRNA expressions seem not to be prerequisite for the phase resetting of the clock in the SCN, because the behavioral phase shift is dissociated from light-induced *per* expression in the PAC_1 knockout mice (*73*). The other component of the circadian feedback loop that was shown to respond to light stimulation is Bmal1 (*40*). The expression pattern of Bmal1 protein is similar to that of Per in *Drosophila*, since both have a peak at midnight and are degraded by light stimulation. However, further analyses are required, because an inconsistent datum was recently reported (*81*).

Output pathways: How is the oscillation in the SCN transmitted to the outside?

The molecular mechanism by which the circadian signal is transmitted from the SCN to other sites as neural activities remains unclear. Recent works using DNA microarray techniques have revealed that many genes exhibit circadian rhythms but relatively few genes might be directly regulated by the Clock:Bmal1 heterodimer (*82*). One possibility is that the Clock:Bmal1 heterodimer might regulate the transcription of a subset of transcriptional factors, such as DBP (*83–85*) and that these enhancers and repressors, such as E4BP4 (*86*), make a transcriptional network and regulate the expression of genes differently. Electrophysiological phenomena, such as resting membrane potentials, may be regulated by the circadian clock mechanism, because a single SCN neuron can exhibit the circadian rhythmicity in its firing frequency (*5*). However, the molecular mechanism involved is completely unknown. The neural activity of the SCN neurons is thought to be transmitted to other areas by humoral factors, because the fetal SCN transplants wrapped in the semi-permeable membrane can restore the rhythmicity in SCN-destroyed animals (*87*). Possible transmitters of the circadian rhythm have been thought to be arginine vasopressin, TGFβ and prokineticin2 (*88*, *89*).

One of the authors recognized that SCN neurons are involved in the control mechanism of glucose metabolism through its action on the autonomic nerves (*90–92*). This suggests that there are neural connections from the SCN to tissues related with glucose metabolism, such as the pancreas, liver and adrenal gland. In fact, experiments recently done by researchers including one of the authors using Pseurorabies virus, which is retrogradely and multisynaptically transported, indicate that the SCN sends neural inputs to all of the peripheral tissues examined, such as the pancreas, white and brown adipose tissues, adrenal, liver, heart, stomach, kidney and thyroid (*93–96*). Therefore, it is quite possible that these autonomic connections from the SCN to peripheral tissues are involved in the generation of circadian rhythms in peripheral tissues through neural outputs from the SCN to them. However, one of the authors also recognized that the autonomic connections from the SCN to peripheral tissues function not only for the synchronization of circadian rhythms in the peripheral tissues, but also for the maintenance of the homeostases of blood glucose, blood pressure and other physiological phenomena essential for the survival of mammals (*90–92*, *97*). That is, homeostatic responses, such as hyperglycemia and sympathetic enhancement due to energy deficiency of the brain, autonomic changes due to illumination of the eye and hypotensive action of L-carnosine, disappeared after bilateral lesions of the SCN. Therefore, these findings raise the possibility that formations and functions of the autonomic nervous system might have been obtained by mammals in the course of evolution of the clock mechanism, thus that the SCN is implicated in homeostatic mechanisms of mammals. Whether this is the case must be examined in future.

Postface

This review compares the fundamental molecular mechanism of the circadian clock in mammals with that in other species. Understanding of the outline of this mechanism has advanced dramatically in the past decade, although many details remain to be clarified, as described above. The mechanism of the mammalian circadian rhythm has been studied for many years using physiological and pharmacological approaches, and recent approaches using biochemistry and molecular biology have resulted in enormous progress in this field. Therefore, further studies including biochemistry, biophysics and bioinformatics in this field may provide a major breakthrough in the understanding of the clock mechanism.

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