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PERIPHERAL CIRCADIAN OSCILLATORS AND THEIR RHYTHMIC REGULATION

Chiaki Fukuhara and Gianluca Tosini

Neuroscience Institute, Morehouse School of Medicine, USA

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1. ABSTRACT

Most of the organisms living on earth show 24 hour (circadian) rhythms that are endogenously controlled by biological clocks. In mammals, these rhythms are generated by the circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus. However, recent studies have demonstrated that circadian oscillators can be found in many organs and tissues, and it appears that the circadian oscillators in the periphery are not self-sustained, since, *in vitro,* the oscillation disappears after a few cycles. Although analysis of the clockwork mechanism indicates that the molecular composition of the clock in the SCN and in the peripheral tissues is very similar, the mechanism responsible for the damping of the circadian oscillation in the periphery is unknown. Recent studies have also indicated that the mammalian circadian system is hierarchically organized in that the SCN (i.e., the master circadian pacemaker) controls the peripheral oscillators in order to coordinate the physiological events in an entire body. The mechanisms by which the SCN controls peripheral oscillators are just starting to be elucidated. The aim of this review is to summarize the most recent findings on functioning of these extra-SCN oscillators and the mechanisms the SCN controls peripheral oscillators.

2. INTRODUCTION

Circadian rhythms in locomotor activity, sleep-wake cycles, hormonal and physiological events within the body have been described for many vertebrates and invertebrates. In mammals, the suprachiasmatic nuclei (SCNs) of the hypothalamus contain the master circadian pacemaker (1) that controls the vast majority of the circadian rhythms. However, experimental evidence gathered over the last 20 years indicates that mammals possess other circadian oscillators outside the SCN. For example, in the retina, circadian rhythms in rod photoreceptor disk-shedding persist after optic nerve section and in the SCN-lesioned animal (2, 3). In addition, several studies have demonstrated that SCN-lesioned animals can successfully anticipate the time of feeding when food is available only at limited times during the day (4). Further evidence to support the idea that mammals have multiple circadian oscillators come after the cloning of the "clock genes." As soon as these genes were cloned, it became evident that they are not only expressed in the SCN, but their expression is widespread within the body. In several of these tissues, in fact, the expression of these genes shows robust circadian rhythms *in vivo* (5).

Although these experimental data strongly suggested the presence of circadian oscillators outside the SCN, they did not prove the existence of functional circadian oscillators in the periphery. In fact, the only way to localize structures that have the capability to oscillate is to maintain that organ *in vitro* while recording a rhythmic output. In the last few years, new *in vitro* preparations have become available, and circadian rhythms in several isolated extra-SCN preparations have been recorded (6-9).

The aim of the present review is to summarize the current knowledge about the location, functioning, and regulation of peripheral oscillators in the periphery.

3. THE CIRCADIAN CLOCK IN THE RETINA

As we have just mentioned, many experimental data suggested that the mammalian retina

contains a circadian pacemaker responsible for control of the retinal circadian rhythms. However, it was only a few years ago that a circadian rhythm in melatonin release was recorded from cultured mammalian retinas (6, 7). This circadian rhythm in melatonin release free-runs in constant darkness for at least 5 cycles; it is entrainable by the light (6, 7), and it is temperature-compensated (10). The observation that cultured retinas of mammals show the three fundamental characteristics of a circadian clock (i.e., free-run, entrainment and temperature compensation) demonstrates the presence of a *bona fide* circadian clock in this tissue.

Although we now know that a circadian clock is located within the neural retina, we still do not know where this clock is located (i.e., which cell types are responsible for the generation of rhythmicity). As a first step to localize the circadian pacemaker within the neural retina, several studies have investigated the expression of "clock genes" in this tissue. In the mouse, *Period1*, *Bmal1* and *Clock* mRNAs are present in the photoreceptors layer, but they are also expressed in the inner nuclear layer and in the ganglion cell layer (11). *Cryptochrome1* and *Crptochromey2* are mostly expressed in the ganglion cell layer (12). In the rat, the available data indicate that *Period1* may be expressed in the photoreceptors, but the vast majority of the transcripts seem to be localized to the inner nuclear layer (13). The same seems to be true for *Period2* mRNA. *Bmal1* and *Clock* transcripts were tentatively localized to the outer nuclear layer (14). Most of these genes show a similar pattern of expression, albeit delayed by about 4 hours, from what has been observed in the SCN. Although these studies have provided useful information, it is evident that the cellular localization of the circadian clock in the retina remains elusive, and further studies are needed to address this important question.

Although the circadian pacemaker in the retina is responsible for generation of the rhythmic events in the retinal physiology (e.g., melatonin synthesis, rod photoreceptor disk shedding, and visual sensitivity), the contribution of the retinal clock to the mammalian circadian organization is not yet established. In this context it is worth mentioning that a recent study has indicated a potential interaction between the retina and SCN in the expression of the circadian rhythm of locomotor activity. In this study, the authors reported that enucleated hamsters showed a broader range of free-running periods than intact hamster suggesting that the SCN may interact with the retina to determine the free-running periods (15).

4. CIRCADIAN OSCILLATORS OUTSIDE THE SCN AND RETINA

In mammals, the molecular mechanism that generates circadian rhythms is based on auto-regulatory transcriptional and translational feedback loops that have both positive and negative elements (16). The positive components are two basic helix-loop-helix, PAS domain-containing transcription factors, CLOCK and BMAL1. These transcription factors drive the transcription of the three *Period* genes (*Period 1, 2, 3*) and of the two *Cryptochrome* genes (*Cryptochrome1* and *2*) through the E-boxes in these genes. The PERIODs and CRYPTOCHROMEs protein dimers act as negative components of the feedback loop, since they inhibit BMAL1:CLOCK-mediated transcription. Recent studies have shown that BMAL1:CLOCK heterodimers activate the transcription of the orphan nuclear receptor gene *Rev-Erb alpha* and then, the REV-ERB alpha proteins repress *Bmal1* transcription by acting through Rev-Erb/ROR response element present in its promoter. Therefore, these data suggest that the positive and negative transcriptional feedback loops are both co-regulated by BMAL1:CLOCK heterodimers (17) (see Figure 1).

As already mentioned, these "clock genes" are expressed in several organs and tissues within the mammalian body (5). In the peripheral tissues (i.e., lung, liver, kidney, muscles etc etc.) the phase relations among the "clock genes" are conserved, but phase is delayed from that observed in the SCN. In the majority of these tissues and organs *Period* genes peak in the late day to midnight, *Cryptochromes* peak in late night, while *Bmal1* peaks light-dark transition, and *Clock* does not show any significant rhythms (5, 12, 18-20). In general, it has been observed that the proteins of each of the "clock genes" show similar patterns, albeit with a few to several hours delay with respect to their mRNA (16).

Although many of these "clock genes" show strong circadian patterns of expression *in vivo*, it is worthwhile to note that such rhythmicity disappears after SCN lesion (20, 21), suggesting that these peripheral tissues and organs are unable to endogenously generate circadian oscillations. However, recent studies dramatically changed this view. In a series of experiments, Menaker and collaborators using a transgenic rat in which the *Period1* promoter has been fused to luciferase gene have demonstrated that many organs and tissues when isolated and cultured may show a circadian oscillation in *Period1*-Luciferase activity (8, 9). Nevertheless, these experiments also indicated that important differences must exist between the SCN and the other circadian oscillators, since the SCN oscillates for many cycles while in the periphery the oscillation persists for only 2-7 cycles (8, 9, Figure 2). An additional study reported that these oscillations can be observed in many parts of the central nervous system such as the pineal gland, pituitary gland, arcuate nucleus, olfactory bulb, median eminence, paraventricular nuclei of the hypothalamus, and supraoptic nucleus (9). However, it is also important to note that some organs and tissues do not show any circadian oscillation at all. For example, *Period1*-Luciferase activity does not show significant rhythmicity in the nucleus accumbens,

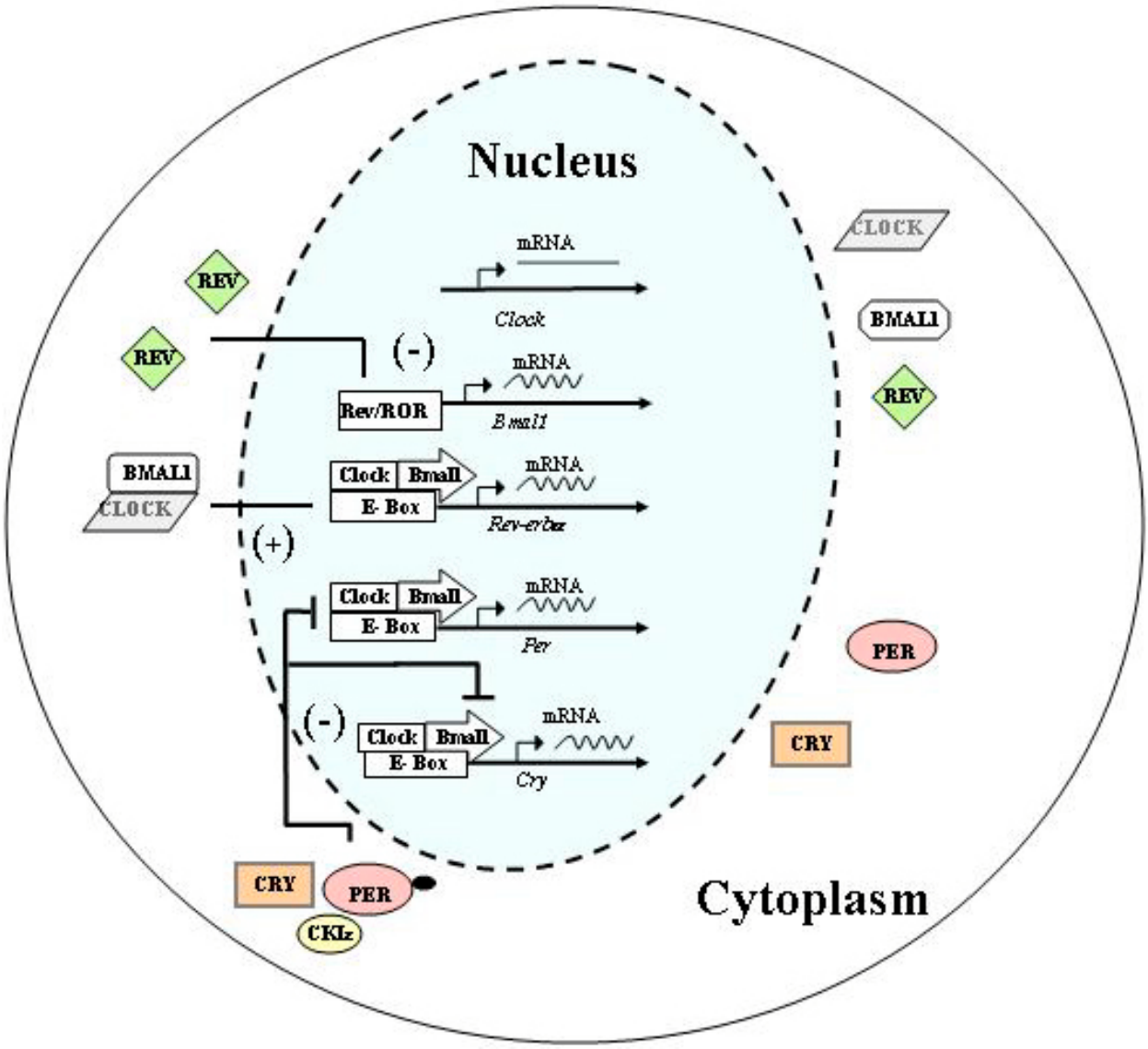

Figure 1. The circadian clock mechanism in mammals. In this model, transcription of Period (1, 2), Cryptochrome (1, 2), and Rev-Erb alpha is positively regulated by the heterodimer formed by the protein products of the Clock and Bmal1 genes. From in vitro experiments, it has been shown that the products of the Period genes and CKI epsilon, CRYPTOCHROME1 and CRYPTOCHROME2 are all able to participate in the formation of complexes in various ways. However, it is not known which complexes are actually formed in vivo. Some or all of these proteins may participate as negative regulators of the transcription of the Period and Cryptochrome genes, although recent evidence implies that PERIOD-CRYPTOCHROME dimers may be the most important in this respect. Bmal1 transcription is inhibited by REV-ERB alpha. In the figure the "minus" sign indicates an inhibiting effect and the “plus” indicates activating effect.

dentate gyrus of the hippocampus, bed nucleus of the stria terminalis, or substantia nigra (9).

One of intriguing aspects of the mechanism of circadian rhythm generation is why only the SCN, but not other structures, can sustain circadian rhythms for a long time. The fact that the phase relationship among the “clock gene” expression patterns in the periphery is similar to that observed in the SCN suggests that the clock mechanism in the peripheral tissues is the same, or very similar, to that seen in the SCN. However, it must be noted that recent studies have also indicated that some differences may exist between the central and peripheral oscillatory mechanisms. For example, in the *Clock* mutant animals, *Bmal1* mRNA levels show no rhythms in the SCN, while they show circadian rhythms, although with reduced amplitude, in the periphery (23). This result suggests that *Clock* is necessary for *Bmal1* rhythmic expression in the SCN but not in the

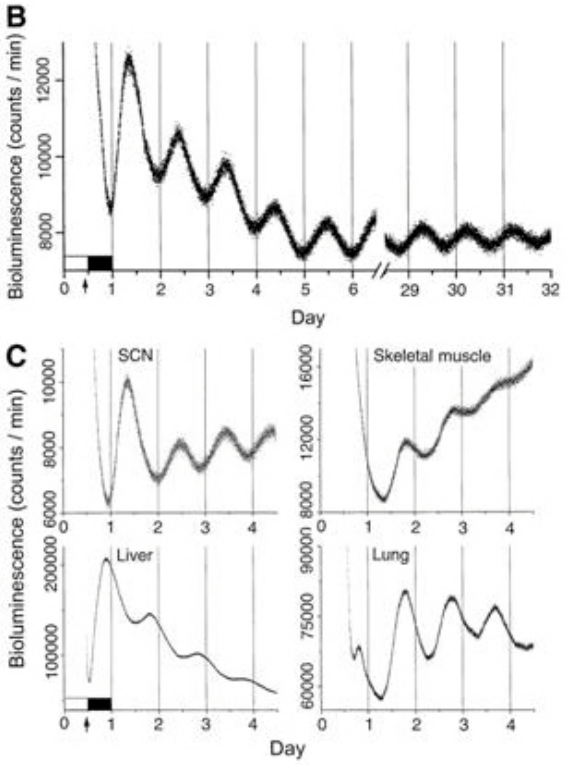

Figure 2. SCN, skeletal muscles, liver and lung explants from transgenic rats expressing *Period1*-driven luciferase reporter gene show circadian rhythms in bioluminescence in culture. The SCN rhythm does not damp in culture, whereas in the other tissues circadian rhythms dampen after a few cycles (from: Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M, and Tei H. Resetting central and peripheral circadian oscillators in transgenic rats. Science 2000; 288: 682-685. Copyright [2000] American Association for Advancement of Science. http://www.sciencemag.org).

periphery. Another example is shown in studies which identified Neuronal PAS domain protein 2 (NPAS2 or MOP4). NPAS2 shares high homology with CLOCK and is expressed in the some peripheral tissues (e.g., vasculature and forebrain), but not in the SCN. NPAS2 may be a component of circadian clock machinery, since it forms heterodimers with BMAL1, promotes E-box-mediated gene expression, and NPAS2 itself is negatively regulated by CRYPTOCHROME1 and 2 (24-30). In addition to that in *Npas2* null mutant mice, the circadian rhythm in *Period2* mRNA is disrupted in the piriform cortex, caudate putamen, and dentate gyrus where *Npas2* is expressed. The fact that *Npas2* is not expressed in the SCN suggests that the product of this gene may be important for the correct functioning of the peripheral clocks.

Additional evidences suggesting the presence of a different mechanism of rhythm generation in the SCN and in the periphery have been provided from a series of investigations in which knockout mice were used. For example, *Period1* mutant mice display a slight change in the free-running period of the locomotor activity rhythm but no significant alteration in the expression of "clock genes" in the SCN (31). However, in these mutant animals the rhythmic expression of "clock genes" in several peripheral tissues is delayed, thus indicating that *Period 1* may play a specific role in the peripheral tissues (31).

From what we have just described, it is clear that there are tissue-specific differences in the molecular composition of the circadian clock, and clock components may play different roles in different tissues. Further analysis of the differential mechanisms of rhythm generation in the central and peripheral oscillators should provide an answer to the question of why the SCN is the only tissue capable of generating self-sustained circadian rhythms.

The damping of the circadian oscillation observed in the peripheral tissues is not due to a deterioration of tissue/organ conditions as results of the culture conditions, since the oscillation can be re-initiated if the tissues or organs are properly stimulated. For example, initiation of circadian rhythms in "clock mRNA" levels following activation of cAMP-dependent protein kinase A (PKA), protein kinase C (PKC), Ca^{2+} , or mitogen-activated protein kinase (MAPK) signaling pathway, has been observed in fibroblasts (32-35) and in the pineal gland (Figure 3). Rhythmic *Period1*-Luciferase activity is re-induced following forskolin stimulation in the arcuate nucleus (9).

Work in our laboratory suggests that initiation of circadian rhythms in peripheral tissue may depend on the signaling pathway that is activated. For example, it has been shown that the pineal gland contains dampened circadian oscillators, but these oscillators need to be driven by the SCN to maintain robust circadian rhythmic expression *in vivo* (9, 36-41). Norepinephrine acutely induces *Period1* expression in cultured pineal (41), but it cannot initiate a robust circadian oscillation in *Period1* mRNA levels, whereas stimulation with cAMP-analog induces robust circadian expression in *Period 1* mRNA (Figure 3). These results indicate that activation of a specific pathway (cAMP) can be more effective than activation of multiple signaling pathways as in the case of NE stimulation.

The mechanisms whereby external stimulation initiates circadian rhythm in fibroblasts (or in any other cells, tissues, or organs) are not completely understood. Akashi and Nishida (35) reported that when a specific MAPK kinase MEK inhibitor U0126 is added to the fibroblasts before ester 12-o-tetradecanolphorbol 13-acetate (TPA) stimulation, circadian rhythms are not initiated, whereas when U0126 is added 8 hours after TPA stimulation, the circadian rhythms are initiated, thus suggesting that MAPK may play an important role in the rhythm initiation.

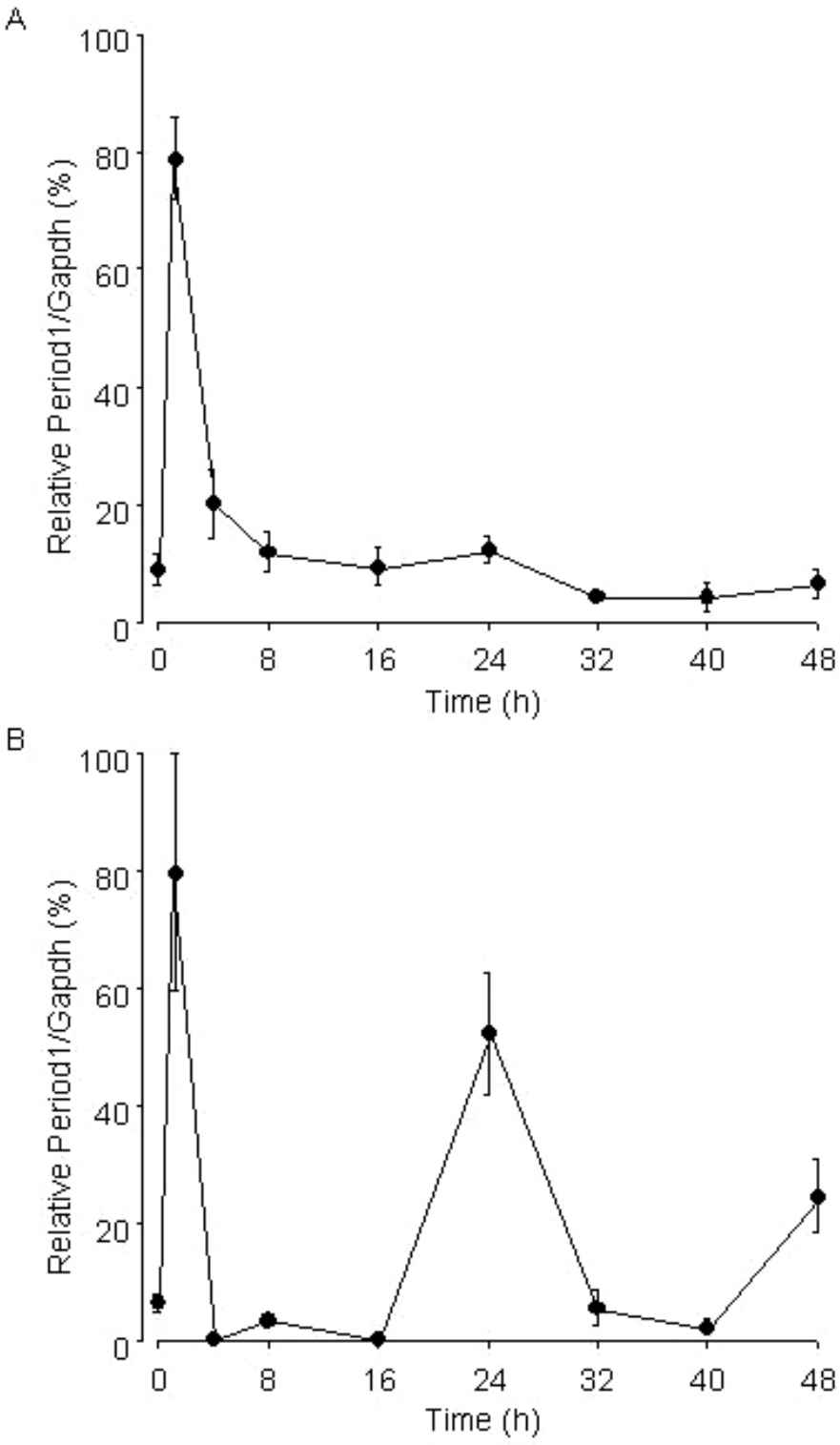

Figure 3. Effect of brief exposure to norepinephrine (NE) (A) and cAMP analog (B) on rhythmic Period1 expression in the cultured rat pineal gland. The pineal glands were cultured for 3 days, stimulated using NE (1 □M) or cAMP analog pCPT-cAMP (1 mM) for 4 hours, and Period1 mRNA levels were measured at indicated times using real time quantitative PCR. NE did not initiate circadian rhythms in Period1 mRNA levels, while pCPT-cAMP initiated robust circadian rhythms ($P < 0.001$, ANOVA). Values are means ± SEM (n= 3-7).

5. REGULATION OF PERIPHERAL OSCILLATORS

Circadian rhythms need to be entrained to environmental cues, and light is the most effective time cue used by the animals to synchronize them to the external environment. In mammals, the light information is only perceived by the retina, and this information reaches the SCN via a distinct neural projection called the retino-hypothalamic tract (42).One of the most intriguing aspects of the mammalian circadian system remains the search for the mechanisms by which the SCN conveys this light information to the rest of the body in order to keep the whole circadian system synchronized. Experimental evidence suggests that both humoral and neural factors are involved in this process. Recent studies have shown that restoration of the circadian rhythms in locomotor activity in the SCN-lesioned animals has been achieved after transplantation of the SCN, which has been encapsulated in a semi-permeable polymeric capsule preventing neural outgrowth but allowing the diffusion of humoral signals (43, 44). The nature of such "diffusible factor" is still unknown, but recent investigations have begun to unveil the possible nature of this elusive factor.

In a screening aimed to identify such factors, Weitz' group discovered that the transforming growth factor-alpha (TGF-alpha) is rhythmically expressed in the SCN, and when TGF-alpha was infused into the third ventricle, it inhibited locomotor activity and disrupted the circadian rhythms of locomotor activity and sleep/wake cycle (45). This result suggests that TGF- alpha is probably one of the diffusible factors responsible for the restoration of the circadian rhythm in locomotor activity (43, 44).

As already mentioned, the possibility of entraining animals to a feeding regimen has been reported by several studies, and two recent papers have demonstrated that in animals subjected to a regimen of restricted feeding (i.e. with food available only at particular times of the day), the circadian oscillators in the liver can be entrained independently from the SCN and the light:dark cycles (46, 47). Abrupt changes in the feeding time leads to a gradual resetting of the rhythmic gene expression, thus indicating that phase resetting is mediated by clock-dependent mechanisms. Interestingly, the food-induced re-synchronization proceeds faster in the liver than in the other peripheral tissues. This suggests that, as in the case of the resetting action of the light on the SCN, different tissues (cells) may preferentially employ specific synchronizing signals.

For example, it appears that glucocorticoids play a role in the entrainment of the peripheral oscillators, since its analog dexamethasone can induce circadian gene expression in cultured rat-1 fibroblasts (48), and systemic injection in the mouse affects the phase of circadian gene expression in the liver, kidney and heart, but not in the SCN neurons (49).

Finally, it is worth mentioning that a potential molecular mechanism by which hormonal factors may affect clock machinery has been reported. Administration of retinoic acid causes phase shifts in the vasculature *in vivo* and in smooth muscle cells *in vitro* (29). Retinoic acid exerts its effect via retinoid receptors, members of the steroid/thyroid hormone receptor superfamily, retinoic acid receptor (RAR) alpha and retinoid X receptor (RXR) alpha. Upon retinoic acid stimulation, these receptors interact with BMAL1:NPAS2 and negatively regulate BMAL1:NPAS2-mediated gene expression.

A recent study has shown that when mouse embryo fibroblasts (MEF) from *Period1* mutant animals are transplanted into the wild-type animals, fibroblasts from *Period1* mutant show circadian rhythms in those periods are similar to those in the wild type. Conversely, when MEF from *Clock* mutant animals are transplanted to the wild-type, circadian rhythms are not rescued in the MEF. The results suggest that there is a functional dependence of the peripheral oscillators with respect to the SCN, since the SCN can rescue or compensate the genetic defects affecting the period of the peripheral clocks (50). It also suggests that functioning circadian oscillators in the periphery are required to express circadian rhythms with the control of the SCN.

Although it has been shown that the mammalian circadian system is hierarchically organized, non-mammalian vertebrate may be organized in a different manner. For example, fishes, reptiles and birds have self-sustained circadian oscillators in the periphery (e.g., retina, pineal gland and parietal eye) that also are directly photosensitive, and therefore their relationship with the SCN is not as dependent as in mammals. Recent studies have also indicated that in the zebrafish the peripheral circadian oscillators in the kidney and heart are also directly photosensitive and can independently entrain to the environmental light:dark cycles suggesting that in this animal a central circadian pacemaker may be not necessary (51, 52).

Interestingly, it has been reported that although the SCN grafts restore behavioral activity rhythms, they fail to restore circadian rhythms in melatonin, serum corticosterone, and cortisol levels as well as gonadal and testicular regressions (43, 53), suggesting that the SCN must employ factors other than humoral to control peripheral rhythmic events. Indeed recent studies have demonstrated that the SCN projects to several organs, including the hypothalamo-pituitary-adrenal axis, the hypothalamo-pituitary-gonadal axis, the hypothalamo-pineal axis, and the hypothalamo-pancreas axis (54-57). These results suggest an active role for neural connections in controlling the expression of circadian rhythms in the periphery (58).

Another intriguing aspect regarding the functioning of peripheral oscillators resides in the observation that different tissues and organs respond in a different way to a phase shift. In a recent study, Yamazaki and colleagues (8) reported that a 6-h phase-shift (delayed or advanced) in light:dark cycles immediately resulted in phase shifts in the *Period1*-Luciferase activity rhythms in the SCN, while skeletal muscles and lung took 6 days to adjust to new light-dark regimens. The results obtained with the liver were even more surprising since phase shift is not achieved after sixteen days (*Period1* oscillation was still delayed about 4 hours). In addition, it appears that phase delays and advances have different effects on the peripheral oscillators. The mechanisms responsible for such differences in the resetting of the circadian rhythms between the SCN and the peripheral tissues are still unknown.

6. CIRCADIAN GENE EXPRESSION IN THE SCN vs PERIPHERAL TISSUES

So far three studies have investigated gene expression profiling using cDNA microarray in the SCN and peripheral tissues over a 24-hour period. One study reported that 9% of the 2122 genes present in the chip showed robust circadian rhythms in the mouse liver (22). Interestingly, they observed that the circadian regulation of the genes was tissue-specific since rhythmic liver genes were not necessarily rhythmic in the SCN. SCN lesion abolished, or severely dampened, the circadian rhythm in the expression of these genes. A similar result was also obtained in the second study (59). In the third study, the authors investigated the circadian expression of 12,488 gene expressions in the mouse liver and heart (60). This study indicated that about 10% of the genes expressed in each tissue showed circadian rhythms, but only a few genes (60) were found to be rhythmic in both organs.

In summary, these studies using cDNA microarray technology indicate that the circadian clock regulates the transcription of many genes in the peripheral tissues, but fewer than forty genes have a common expression pattern among the different tissues and organs.

7. PERSPECTIVE

The findings obtained in the recent years have challenged our knowledge about the circadian organization of mammals. It is now firmly established that in mammals several organs and tissues, besides the SCN, can generate endogenous circadian rhythms. As we have already mentioned, one of the most fascinating and unsolved questions in the circadian field is why only the SCN neurons can generate endogenous and self-sustained rhythms that persist for long term. Recent studies have shown different impacts of a series of “clock gene” knockouts on circadian gene expression and locomotor activity, suggesting that clock machinery may differ between the central pacemaker and peripheral oscillators.

The primary role of central circadian pacemaker is to coordinate the phase of the peripheral circadian oscillators so that physiological events can be fine-tuned to the varying environmental demands. However, the physiological role(s) that these peripheral oscillators play in the organs and tissues, and their contributions in the overall circadian organization, is not yet elucidated. Another pivotal question that remains to be addressed is the reason why the rate of entrainment differs among the different peripheral oscillators and the SCN.

There is growing appreciation that the feeling of well-being, alterations in mood, and susceptibility to a variety of medical disorders depend on the proper expression of the master circadian clock and the synchrony among the other oscillators found in many peripheral tissues. These recent findings as well as the ones that will come out in the future years will be instrumental in helping to resolve some of the health problems caused by jet lag, circadian-based sleep disorders, shift work and some neuropsychiatric illnesses.

8. ACKNOWLEDGMENT

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Send correspondence to: Chiaki Fukuhara, Ph.D., Neuroscience Institute, Morehouse School of Medicine, 720 Westview Dr. SW, Atlanta, GA 30310-1495, Tel: 404-756-6697, Fax: 404- 752-1041, E-mail: fukuhac@msm.edu