Circadian Rhythms

3. Circadian Timing Systems: How are they Organized?

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Circadian oscillator, peripheral oscillators, desynchronization, SCN, multi-oscillatory,.

Early in the course of circadian rhythm research, it became clear that individual animal subjects exhibit multiple rhythms whose peaks or troughs assume a fixed temporal relationship with each other, maintaining a state of internal synchrony. These observations motivated enquiry into what could be the nature of the system underlying such rhythms and inspired one of the pioneer circadian biologists, Colin Pittendrigh to develop the concept of circadian organization. Pittendrigh proposed that multiple oscillators constitute circadian timing systems, and each of them regulates a different rhythm. In this article, we will discuss what the evidence over the years suggests about the circadian organization and how our understanding of this system has matured.

Circadian Organization

De Marian's observation that daily rhythms in plant leaf movement continue without the exposure to external light/dark cycles is considered to be the first quasi-scientific investigation of daily rhythms in any living organism. For nearly 200 years following this observation, the study of leaf movements served as the only means to investigate daily rhythms, till its first demonstration in animals/metazoans, in the year 1900. Within 50 years, rhythms in several behaviours and physiological processes were discovered in a wide range of animals.

The study of circadian rhythms in animals revealed that individuals display multiple rhythms. For example, mammals exhibit rhythms in locomotor activity, drinking, body temperature, blood sugar, liver glycogen, eosinophil count, adrenal activity, pineal melatonin and corticosteroid levels and sensitivity to drugs. Moreover, different rhythms have their peaks and troughs at

different times of the day, such that individual rhythms attain a specific temporal relationship with environmental cycles and also with other rhythms leading to a state of 'internal synchrony'. These features have been the inspiration behind the enquiry into two key issues related to circadian rhythms – (1) the nature of the system that underlies such rhythms and (2) the usefulness of these rhythms to living beings. As far as usefulness of circadian rhythms is concerned, internal synchrony among multiple rhythms is thought to coordinate internal physiology, and thereby confer benefits to living beings. In this article, we will concentrate on the first of the two issues, which enquires about the nature of the systems underlying internal synchrony and their temporal relationship with the environment, which is referred to as 'circadian organization'.

Pittendrigh, in one of his seminal contributions to the field of circadian biology, hypothesized a system underlying the regulation of multiple rhythms, based on the evidence for the oscillatory nature of the rhythms (*Box* 1) and observations available then, that multiple tissues or organs of mammalian systems are capable of maintaining self-sustained near 24 h rhythms in laboratory cultures. He proposed that circadian systems comprise of a population of oscillators, each one governing a different rhythm. Years of research on metazoan systems ranging from invertebrates to mammals have shown that circadian timing systems are indeed composed of self-sustained oscillators residing in various tissues, and thus are thought to regulate rhythmic functions associated with the respective tissues.

Here, we review the development of our understanding of circadian organization in metazoans, where we will visit the evidence that has demonstrated the multi-oscillatory nature of circadian timing systems. Although, it appears that multi-oscillatory systems are a universal feature of circadian organization, animal systems differ widely in the way the component oscillators are organized to achieve internal synchrony and to schedule behaviours and physiological functions at the appropriate time of the day.

Previous articles:
1. Koustubh M Vaze and Vijay Kumar Sharma, 'From Daily Rhythms to Biological Clocks', *Resonance,* Vol.18, No.7, pp.662–672, 2013.
2. Nikhil K L and Vijay Kumar Sharma, 'The Underlying Molecular Mechanisms', *Resonance,* Vol.18, No.9, pp.832–844, 2013.

Box 1. Circadian Rhythms as Biological Oscillators

By the 1960s, the study of circadian rhythms in animal models ranging from invertebrates to mammals along with those in plants and some unicellular organisms had established several generalizations about its properties.

Some of the key features of circadian rhythms are as follows:

1) These rhythms are innate, endogenous and self-sustained. Under constant conditions, they free-run with an endogenous period which is close to but seldom equal to 24 h. (Vaze and Sharma, *Resonance*[1]).

2) The period of these rhythms are unaffected by temperature changes within physiologically permissible limits (15–35 ^{o}C). This property of circadian rhythms is known as temperature compensation.

3) These rhythms are capable of synchronizing to cyclic environmental variables such as light and temperature. This process is known as entrainment.

4) The phase of the free-running circadian rhythms can be shifted (advanced/delayed) by exposure to brief pulses of light or temperature, and the magnitude and direction of such phase-shifts depend on (a) the phase of the rhythm and (b) the strength of the pulse. Such phase-dependent effects of light or temperature pulses are often represented as phase response curves (PRCs).

5) Phase-shifts elicited by light or temperature pulses may occur over several cycles before the rhythm attains a new steady-state and the intermediate cycles where the phase of the rhythm continues to drift before reaching the steady-state are known as transients.

By the late 1950s, Colin S Pittendrigh and his colleague Victor Bruce observed[2] that the properties of circadian rhythms especially their endogenous, self-sustained nature, ability to entrain to environmental cycles and PRCs, greatly resemble those of self-sustained physical oscillators; which led them to propose that circadian rhythms function like physical oscillators and named such biological oscillators as 'endogenous self-sustained oscillators (ESSOs)' – in contemporary literature more commonly referred to as 'circadian oscillators'.

It is important to note that the proposition that oscillator-like systems underlie circadian rhythms was an inference based on the resemblance between the properties of biological rhythms and physical oscillators. Although an oscillator-like system was hypothesized to underlie circadian rhythms, at that point of time there was hardly any information about the physical nature of the system. However, the idea of circadian rhythms as oscillators provided a unifying framework to visualize mechanisms underlying circadian rhythms, which has been eventually shown to be a reality.

[1] Koustubh M Vaze and Vijay Kumar Sharma, 'From Daily Rhythms to Biological Clocks', *Resonance,* Vol.18, No.7, pp.662–672, 2013.

[2] C S Pittendrigh and V G Bruce, An oscillator model for biological clocks, In *Rhythmic and Synthetic Processes in Growth*, D Rudnick (Ed.), pp.75–109. Princeton University Press, Princeton, 1957.

Evidence for Multiple Circadian Oscillators

Circadian timing systems consist of multiple oscillators.

Evidence for multiple circadian oscillators can be broadly classified into two categories. One class of evidence is based on observations of multiple overt rhythms in individual animal subjects. As rhythmic behaviours or physiological processes can be considered to be an overt expression of the underlying circadian oscillators, differences in the properties of the overt rhythms exhibited by individual animal subjects has been taken as evidence for the presence of multiple oscillators.

The efforts to identify the site(s) of circadian oscillator(s) have yielded direct evidence for the presence of self-sustained circadian oscillators (SSCO) at multiple anatomical locations. In the early days of circadian research, the brain/central nervous system (CNS) was thought to be the seat of circadian clocks and therefore initial efforts to locate circadian oscillators were directed towards parts of the CNS. The identification of a part of the brain as a potential site of circadian oscillators was based on the simple principle of demonstration of 'necessity' and 'sufficiency' of that part of the brain for the expression of overt circadian rhythms such as locomotor activity rhythm. Localised tissue lesions (which abolish their function) and tissue transplantation were used to test the necessity and sufficiency of the tissue in the regulation of overt circadian rhythms. Disruption of the rhythm upon tissue lesion indicated the necessity and restoration of the rhythm by transplantation of intact tissue suggested the sufficiency, of that tissue for regulation of the rhythm. Although, tissue lesions and transplantation can suggest the probable site of circadian oscillators, it cannot rule out the possibility that the tissue under investigation is merely a carrier of rhythmic information from the oscillator situated elsewhere. Therefore, demonstration of persistent rhythmic output from the tissue, under constant conditions is essential to establish that the tissue under investigation is the seat of circadian oscillator. This was achieved by recording neuronal activity or other rhythmic outputs from the tissue sections, completely isolated from the rest of the brain, or from tissues maintained in cultures. Thus, the anatomical locations of circadian oscillators

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have been identified based on three types of evidence – (1) arrhythmicity or changes in the properties of overt rhythms upon destruction of tissue/organ of interest, (2) restoration of the rhythms by transplantation of intact tissue/organ from other individuals and (3) persistence of circadian rhythms under *in vitro* tissue/organ cultures.

Advances in our understanding of the molecular bases of circadian rhythms further facilitated the localization of circadian oscillators (*Box* 2). Identification of genes whose products

Box 2. Molecular Bases of Circadian Rhythms: Implications for the Analyses of Circadian Organization (Nikhil and Sharma, *Resonance*[1])

The significance of the genetic and molecular analyses of circadian rhythms in fruit flies *D. melanogaster* lies in the fact that it revealed the physical nature of the circadian oscillators proposed by Pittendrigh and Bruce, which was earlier only perceivable through the properties of the overt rhythms. However, this analysis also had radical implications for the understanding of many other aspects of circadian rhythms, and circadian organization is certainly one of them. *Drosophila period* (*per*) gene became known due to the discovery of mutations which caused arrhythmicity or altered locomotor activity and adult emergence rhythms, by Ron Konopka and Seymour Benzer from California Institute of Technology in 1971. The per^{01} mutant was arrhythmic whereas per^{S} and per^{L} showed short (19 h) and long (29 h) periods respectively of locomotor activity and adult emergence rhythms[2].

Characterization, cloning and sequencing of the *Drosophila per* locus established the functional importance of gene products in the regulation of circadian rhythms. Further studies also revealed that cell autonomous negative feedback loop involving the PER protein on its own transcription constitutes *Drosophila* circadian oscillators. Subsequently, a group of lateral neurons in cerebral lobes showing rhythmic expression of PERIOD protein were found to be necessary and sufficient for the expression of circadian rhythms, leading to the identification of oscillator neurons in *Drosophila*. Over the next few years, a few more genes coding for oscillator components were identified in *Drosophila* and their homologues in many other vertebrate animal systems such as zebra fish, mouse and rat. Identification of genes coding for components of the core circadian oscillator provided a simple method of localizing autonomous circadian oscillators in other body tissues, as it was based on detection of rhythmic expression of circadian genes without the need to know the probable rhythmic behaviours or physiological functions exhibited by the tissues. As a result, this method soon revealed the presence of autonomous circadian oscillators in several peripheral tissues, strengthening the evidence for multi-oscillatory circadian organization.

[1] Nikhil K L and Vijay Kumar Sharma, 'The Underlying Molecular Mechanisms', *Resonance,* Vol.18, No.9, pp.832–844, 2013.
[2] R J Konopka and S Benzer, Clock mutants of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA*, Vol.68, pp.2112–2116, 1971.

constitute circadian oscillators provided a simple means of detecting these oscillators in various body tissues. Again, analysis of necessity and sufficiency of the expression of such genes in parts of the tissues for the presence of overt rhythms, revealed the precise locations of circadian oscillators, especially in the case of small insects such as fruit flies *Drosophila melanogaster*, where localization of circadian oscillators using traditional lesion and transplantation protocols was limited due to their small size. The circadian expression of mRNA and protein products of these genes were thus used as markers of circadian oscillators.

A group of lateral neurons in cerebral lobes showing rhythmic expression of PERIOD protein were found to be necessary and sufficient for the expression of circadian rhythms, leading to the identification of oscillator neurons in *Drosophila*.

Indirect Evidence

***Internal Desynchronization*:** Individual animals exhibit circadian rhythms in various behaviours and physiological processes and usually the phases of these rhythms, such as peak or trough, occur at fixed times of the day, every day. Multiple rhythms in such a state are said to be in internal synchrony. Two or more rhythms in synchrony would thus exhibit the same period even if they differ in the timings of their peaks or troughs. Desynchronization of circadian rhythms i.e., persistence of different rhythms with different periodicities, would indicate regulation by different oscillators and thus, desynchronization of rhythms has been taken as evidence for the presence of multiple circadian oscillators.

The work of Jürgen Aschoff [1] (another pioneering figure in the field of circadian biology) on human subjects and Sulzman *et al* [2] on squirrel monkeys provide some of the most convincing demonstrations of internal desynchronization. In a study on human subjects kept in isolation, deprived of any information of local time, analysis of rhythms revealed that the period of their locomotor activity and urinary potassium content rhythms lengthened spontaneously following a period of ~33 h (filled and empty circles), whereas their body temperature and water excretion rhythms continued with a period of ~25 h (filled and empty squares) (*Figure* 1). In this study, internal desynchronization was observed only in a subset of the subjects. Since human subjects can voluntarily delay their bed time, it was not clear whether desynchronization of a

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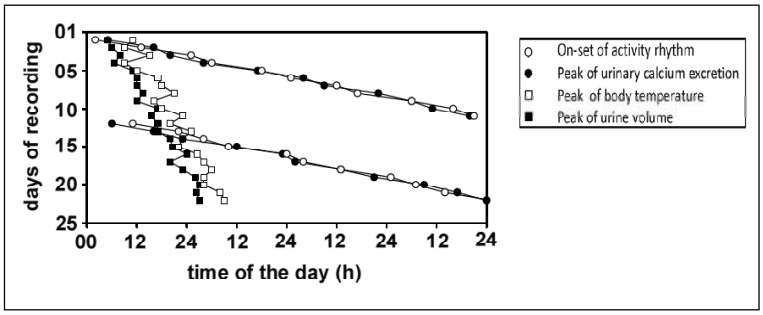

Figure 1. Desynchronization of circadian rhythms in human subjects under constant conditions. Figure modified after Aschoff [1].

group of rhythms could be attributed to the presence of separate circadian oscillators, or to the ability of some rhythms (e.g., urinary potassium) to entrain to locomotor activity rhythm and inability of other rhythms (e.g., body temperature and water excretion) to catch up with it. Similar internal desynchronization was also observed in chair-restrained squirrel monkeys maintained under constant illumination and deprived of any time-cues [2]. In this case, rhythms of feeding, drinking and body temperature showed a shorter period compared to that of urinary potassium rhythm.

***Splitting of Rhythm*:** The two examples discussed above demonstrate desynchronization of different circadian rhythms, but there are also examples of a rhythm getting divided into two or more components. Such a phenomenon is known as 'splitting'. For example, the splitting of locomotor activity rhythm in rodents upon change in the intensity of constant illumination has been extensively studied, and splitting under such conditions has been found to take different forms. In some cases, the activity simply splits into two or more components, each maintaining a fixed temporal relationship with the other, without any change in the period (*Figure* 2, left panel), whereas in other cases fragmented activity components assume distinctly different periodicities (*Figure 2*, right panel). The presence of rhythm components with different periodicities has been taken as an evidence for multi-oscillatory circadian organization (*Figure* 2).

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Internal desynchronization and splitting of rhythms thus provide strong suggestive evidence for multi-oscillatory organization of circadian timing systems.

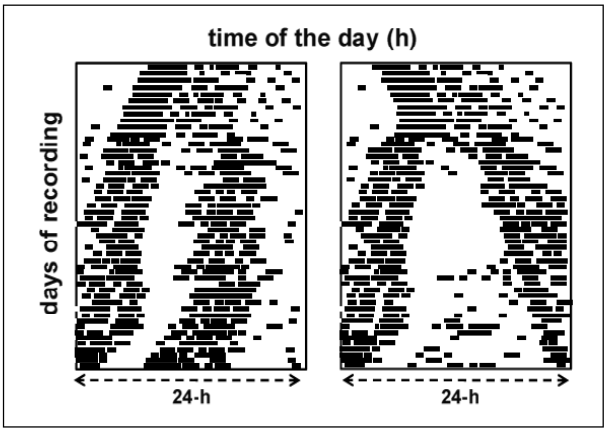

Figure 2. Splitting of locomotor activity rhythms under constant light in hamsters. Left panel shows splitting of activity phase into two components with similar period and right panel shows two components with distinct periodicities. Figure modified after Pittendrigh [3].

Direct Evidence

Direct evidence for multi-oscillatory circadian organization has been a combined result of (1) the early efforts to localize the central circadian oscillators which targeted brain/CNS and (2) the discovery of circadian oscillators in tissues outside the CNS; made possible by progress in understanding of the molecular bases of circadian rhythms, in the 1980s and 1990s.

The earliest efforts to locate circadian oscillators go back to the studies in the 1950s by an insect physiologist from Cambridge, Janet Harker [4], who pioneered the use of tissue lesion and transplantation approach on cockroaches by studying their effects on the locomotor activity rhythm. Parts of the brain tissue, where lesions abolished the locomotor activity rhythm and transplanted tissue from another individual restored the rhythm were identified as the putative sites of the circadian oscillators. This approach has become the guiding principle for studies aimed at identifying circadian oscillators not just in cockroaches but in a range of other animal models.

The earliest efforts to locate circadian oscillators go back to the studies in the 1950s by an insect physiologist from Cambridge, Janet Harker.

Although Janet Harker could not locate the circadian oscillators, it inspired many researchers to take up this challenge. Niishiitsutsuji-Uwo, a postdoctoral fellow in Pittendrigh's laboratory found that surgical ablation of the optic lobes disrupted circadian rhythm of locomotor activity in the cock-

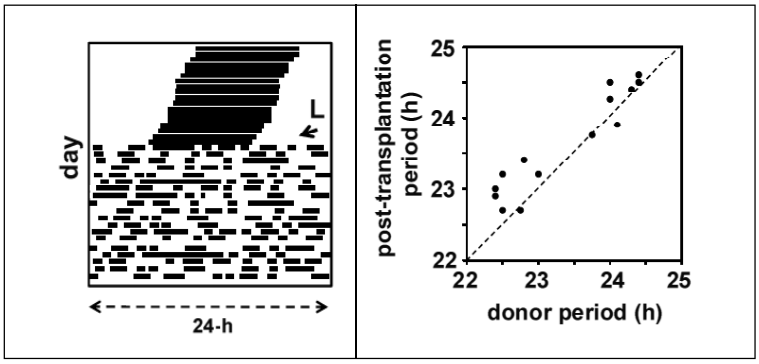

Figure 3 (left). Loss of locomotor activity rhythm following optic lobe lesion in cockroach. Arrow with 'L' indicates the day of optic lobe lesion. The *x*-axis represents time of the day whereas *y*-axis indicates the day of locomotor activity recording.

Figure 4 (right). Correlation between free-running period of locomotor activity rhythms of cockroaches after transplantation with optic lobes from donor individuals and that of optic lobe donor. Positive correlation indicates that post transplantation host individuals assume a free-running period exhibited by optic lobe donor individual thus showing that optic lobe harbors circadian oscillator in cockroaches. (Figure modified after Page [6].)

roach *Leucophaea maderae* [5] (*Figure* 3). Following Niishiitsutsuji-Uwo's observations, studies in many other species of cockroaches and crickets showed loss of circadian locomotor activity rhythm upon the ablation of optic lobes, indicating optic lobes as the probable site of circadian oscillators. Their role as circadian oscillator was further demonstrated in a series of transplantation and organ culture studies. In experiments performed by Terry Page, optic lobes were reciprocally transplanted between individuals with distinctly different period of locomotor activity rhythm. This study revealed that optic lobe transplantation not only restored the rhythm but hosts assumed the period of the donor individuals (*Figure* 4). The demonstration of persistence of circadian rhythms of neural activity in optic lobes isolated from the rest of the brain, or in organ cultures further showed that it is indeed the seat of circadian oscillators. Although, optic lobes were found to be necessary and sufficient for the expression of circadian rhythms in cockroaches and crickets, similar studies in many other insects including fruit flies *D. melanogaster* revealed that optic lobe lesions do not affect their circadian rhythms. Subsequent studies indicated cerebral lobes as the possible site of circadian oscillators; however, the exact location of it was known only after the analysis of spatial expression of the *period* (*per*) gene in *Drosophila* brain, whose mRNA and protein products were found to be essential for the expression of locomotor activity rhythm.

Identification of *suprachiasmatic nucleus* (SCN), a region in the anterior hypothalamus of the mammalian brain, as the central

circadian oscillators of mammals is one of the best examples of the successful usage of lesion approach (*Box* 3). Loss of circadian

Box 3. Suprachiasmatic Nucleus (SCN) – Master Circadian Oscillator of Mammals

Although, SCN was described as early as the1920s, its role as the site of the mammalian circadian oscillator remained unknown until 1970s, when suggestive evidence from different sources led circadian biologists to investigate its role as the master circadian oscillator – (1) Light is known to be the principle entraining cue and hence the location of circadian oscillators was believed to be associated with the light-sensing nervous tissues. By the 1970s, several lines of evidence had shown that projections from the retinal axons (eye tissue) terminate in the anterior hypothalamus. Therefore, the neural connection between retina and hypothalamus – the *retinohypothalamic tract* (RHT) was thought to be the carrier of light information to the circadian oscillator. (2) At the same time, Curt Richter, a neuropsychologist from John Hopkins, had narrowed down to anterior hypothalamus as the potential site of mammalian circadian oscillator controlling locomotor activity rhythm in rats, after extensive studies on the effects of metabolic, endocrine and neurological processes on the activity rhythm. He found that lesions in the anterior hypothalamus disrupts the locomotor activity rhythm. Following these observations, two studies published in the year 1972 demonstrated the loss of circadian rhythms in locomotor activity, drinking and circulating levels of adrenal corticosteroid upon the removal of the SCN[1,2].

Although disruption of behavioural and physiological circadian rhythms upon the removal of SCN strongly indicates SCN as a probable site of mammalian circadian oscillator, the possibility of the SCN's role as mere carrier of rhythmic information from the true pacemaker situated elsewhere could not be ruled out. A set of subsequent studies clearly ruled out this possibility by detecting rhythmic neuronal activity as output. In a landmark study in 1979 by Inoyue and Kawamura[3], *in vivo* recording of neuronal activity from the SCN tissues, completely cut-off from the rest of the brain, continued to exhibit rhythm, while the rest of the brain showed arrhythmic neuronal activity and the animal too showed arrhythmic locomotor activity. Moreover, subsequent studies demonstrated persistence of circadian rhythm in neuronal activity in *in vitro* cultures of isolated SCN tissues. These studies clearly demonstrated that signals for rhythmic output visible in the form of locomotor activity or neuronal firing are generated within the SCN, and thus provided evidence in support of the notion that SCN is the master circadian oscillator in mammals.

Unequivocal evidence for the role of SCN as mammalian circadian oscillator finally came from SCN transplantation study in golden hamsters, which made use of the short period mutant line which was discovered at that time. In a brilliantly performed experiment, SCN tissues from the short period mutant animals were transplanted into the SCN-lesioned wild-type hamsters and *vice versa*. As one would expect, the locomotor activity records showed that the period of the locomotor activity rhythm was determined by the genotype of the transplanted SCN and not of the host (*Figures* A, B, C).

[1] F K Stephan and I Zucker, Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions, *Proc. Natl. Acad. Sci. USA*., Vol.69, pp.1583–1586, 1972.
[2] R Y Moore and V B Eichler, Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat, *Brain Research*, Vol.42, pp.201–206, 1972.
[3] S T Inouye and H Kawamura, Persistence of circadian rhythmicity in a mammalian hypothalamic "island" containing the suprachiasmatic nucleus, *Cold Spring Harbor Symposium Quantitative Biology*, Vol.76, pp.5962–5966, 1979.

Box 3. Continued...

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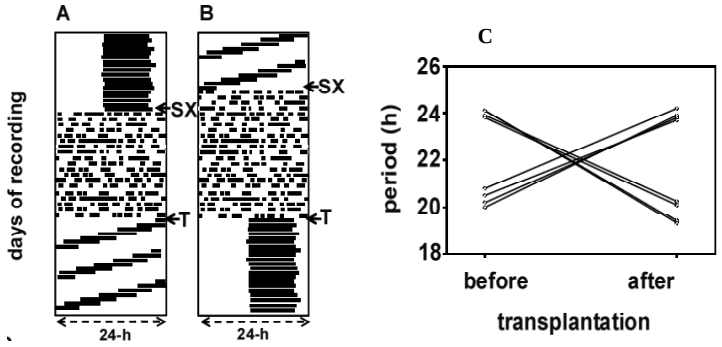

Figures A, B and C. Effect of reciprocal SCN transplantation between wild-type and short period mutant hamsters on the free-running period of locomotor activity rhythms. Wild-type hamsters exhibit a free-running period of ~24 h and short-period mutant has a period of ~20 h. **(A) and (B)** are the representative actograms of wild-type and short period mutant hamsters transplanted with SCN from mutant and wild-type hamsters respectively. Arrows labeled with 'SX' indicate the day of SCN lesion and arrows labeled with 'T' indicate the day of SCN transplantation. **(C)** shows the period of locomotor activity rhythms of hamsters before and after SCN transplantation. Each line in figure C join points indicating the period of the locomotor activity rhythms of individual hamsters before and after SCN transplantation. (Figures modified after Ralph *et al.*[4])

[4] M R Ralph, R G Foster, F C Davis and M Menaker, Transplanted suprachiasmatic nucleus determines circadian period, *Science*, Vol.247, pp.975–978, 1990.

rhythms following SCN lesion demonstrated the necessity of SCN, whereas restoration of rhythms by SCN transplanted from other individuals clearly showed that intact SCN is sufficient for the persistence of circadian rhythms. Moreover, demonstration of circadian rhythms in neural activity of SCN isolated from the rest of the brain confirmed that SCN harbours the autonomous circadian oscillators.

Loss of circadian rhythms following SCN lesion demonstrated the necessity of SCN, whereas restoration of rhythms by SCN transplanted from other individuals clearly showed that intact SCN is sufficient for the persistence of circadian rhythms.

The lesion and transplantation approach have also indicated the anatomical location of circadian oscillators in non-mammalian vertebrates (NMVs) such as birds, reptiles, amphibians and fishes. Evidence suggests that the pineal gland, eye and SCN are the sites of circadian oscillators in NMVs. The pineal gland and the eye were thought to be the probable sites for circadian oscillators because of their association with daily rhythms, whereas the role of mammalian SCN in the regulation of circadian rhythms prompted inquiry into its role in the NMVs. Nearly

all vertebrates possess a pineal gland, the primary source of the hormone melatonin, which exhibits robust daily variation with high levels during the night and low levels during daytime. Moreover, the activity of a rate limiting enzyme in melatonin biosynthesis, N-acetyl-transferase also exhibits daily rhythm, which is why the pineal gland was tested as a potential circadian oscillator for NMVs. On the other hand, studies had demonstrated daily rhythms in eye morphology and physiology such as movement of rods and cones, pigment migration, enzyme activity and melatonin levels in several vertebrates and these were also found to persist under constant conditions. An endogenous rhythm exhibited in the eye tissue was probably the reason to test eyes as the potential site of circadian oscillators. Surgical removal of the pineal gland or eyes in several species of NMVs has revealed a range of effects on circadian rhythms such as period change, splitting and arrhythmicity.

Pinealectomy (surgical removal of pineal gland) in house sparrows induces arrhythmic activity, and transplantation restores the rhythm. Similarly, removal of eye disrupts circadian rhythms of locomotor activity and body temperature in Japanese quails and hence suggest the presence of functional circadian oscillators in these tissues. Like in mammals, localised lesion of SCN disrupts circadian rhythms in fish, lizard and birds. Taken together, this evidence suggests that circadian organization in NMVs is also multi-oscillatory in nature. However, differences in the effects of tissue removal on circadian rhythms in birds, reptiles and fishes, indicate variation in the relative importance of the circadian oscillators in the pineal gland, eyes and SCN.

The classical approach characterised by tissue lesion and transplantation thus localized circadian oscillator(s) to the brain/CNS in animals ranging from insects to mammals. These circadian oscillators were found to regulate rhythms in some of the key behaviours and physiological processes, such as locomotor activity, body temperature and hormonal rhythms, which creates a general impression that CNS harbours oscillators that regulates all the circadian rhythms.

Although there was a widely held notion that the master circadian oscillators regulating circadian rhythms are situated in the CNS, the observations of persistence of circadian rhythms in laboratory cultures of non-nervous system tissues suggested the presence of autonomous circadian oscillators in many tissues outside the CNS. Circadian oscillators found in these peripheral tissues were eventually named as – peripheral oscillators. For example, persistence of circadian rhythm of contraction in isolated intestinal segments, and in cell division, were some of the earliest reports indicating the presence of peripheral oscillators. Many other studies reported circadian rhythms of corticosteroid production in cultures of adrenal glands, of oxygen consumption in liver cells and of enzyme activity in cultures of red blood cells indicating the presence of peripheral oscillators. In several moth species, persistence of circadian rhythm in sperm release from *in vitro* cultures of testes provided evidence for the presence of brain-independent circadian oscillators. Despite ample evidence indicating the presence of peripheral oscillators, their significance in circadian organization was recognized only after the demonstration of their prevalence, made possible by progress in molecular genetics of circadian rhythms in fruit flies *D. melanogaster* (*Box* 2).

Genetic and molecular analyses of mechanisms underlying circadian rhythms during the 1980s and 1990s revealed genes whose products constitute circadian oscillators in *Drosophila*. This also facilitated the identification of homologues in many other vertebrate systems such as zebra fish, mouse and rat. Owing to the role of these genes in circadian functions, their rhythmic expression was soon exploited as the marker of circadian oscillators, which revealed the presence of functional circadian oscillators in several tissues outside the brain. Demonstration of free-running rhythm in PERIOD (PER) protein expression in the Malpighian tubules of *D. melanogaster* is considered as one of the first studies to report tissue autonomous circadian oscillators using this approach. Another study demonstrated rhythm in PER expression in the prothoracic gland of fruit flies. Following this, a number of studies reported circadian oscillators in several non-

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neural tissues in *Drosophila*, zebra fish, mouse and rat. A study by Plautz *et al* [7], demonstrated rhythmic expression of PER in laboratory cultures of isolated proboscis, antennae, legs, wings, eyes, Malpighian tubules and testes of *Drosophila*. Another study by Zylka *et al* [8] reported rhythmic expression of mouse homologues of the *Drosophila per* gene in liver, skeletal muscles and testes. A study by Whitmore *et al* [9] followed the expression of newly cloned gene *clock* in zebra fish and found its expression to be rhythmic in several peripheral tissues including kidney and heart.

Multiple oscillators are organised differently in different species.

The so-called 'master' oscillator identified by traditional lesion/ transplantation approach, and recently discovered peripheral oscillators in animal systems spanning insects to mammals, clearly demonstrate that circadian organization in metazoans comprises populations of self-sustained circadian oscillators.

Organization of Multiple Oscillators

Before the prevalence of peripheral oscillators was recognized, it was thought that the master circadian oscillators situated in the brain regulate all rhythmic behaviours and physiological functions by sending out signals to the peripheral tissues. However, recent evidence for the presence of peripheral oscillators (sometimes autonomous) in virtually every tissue outside the CNS has revealed the complexity associated with circadian organization. Peripheral oscillators are thought to regulate timing of rhythmic tissue-specific behaviours or physiological functions. The fact that multiple oscillators constitute circadian timing system led to the development of models explaining several possible ways in which multiple oscillators may organize themselves to achieve internal synchrony and phasing of biological functions at appropriate time of the day. The hierarchical model (*Figure* 5A) of circadian organization proposes the master oscillator to set the phase of the peripheral oscillators, whereas the non-hierarchical model (*Figure* 5B) assumes that entrainment to environmental cycles and the state of internal synchrony is achieved through the ability of each oscillator to send and receive phase information to

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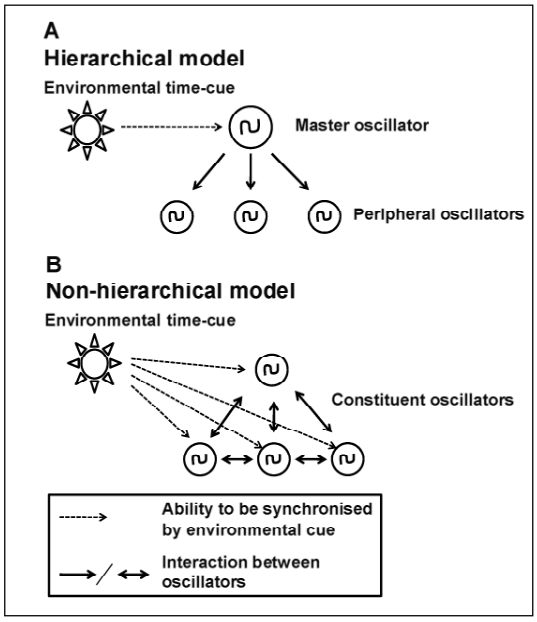

Figure 5. Models of circadian organization.

and from others. Moreover, circadian organization may vary with respect to the possibility of environmental time-cues to directly entrain individual oscillators. Following the discovery of peripheral oscillators, several studies have analysed the circadian organization in different animal models and evidence thus far suggests the presence of both types of circadian organizations.

Monitoring of *period* gene expression in the SCN, liver, skeletal muscles and lungs, before and after the shifts in light/dark cycles revealed that SCN rhythms shift almost immediately, whereas rhythms in the peripheral tissues shift at a rate much slower than the SCN, and different tissues shift at different rates.

Mammalian systems exhibit hierarchical circadian organization consisting of light entrainable oscillators in the SCN which synchronizes peripheral oscillators and hence the rhythms regulated by them. A study by Yamazaki *et al* [9] was the first of its kind to test if self-sustained peripheral oscillators can be entrained by the oscillators in the SCN. It was predicted that if SCN is the synchronizer of peripheral oscillators then sudden advance or delay of light/dark cycles would lead to a temporary desynchronization among the SCN-driven rhythms and in various peripheral tissues, owing to differential responses of peripheral oscillators to the entraining signals from the SCN. Monitoring of *period* gene expression in the SCN, liver, skeletal muscles and lungs, before and after the shifts in light/dark cycles revealed that SCN rhythms shift almost immediately, whereas rhythms in

the peripheral tissues shift at a rate much slower than the SCN, and different tissues shift at different rates. A few years later, a study by Yoo *et al* [11] provided more compelling evidence in support of the hypothesis that the SCN synchronizes rhythms in the peripheral tissues. If SCN synchronizes the rhythms in peripheral tissues, then disruption of SCN should lead to desynchrony among the peripheral oscillators. The study indeed found desynchrony of rhythms in the peripheral tissues within and among the animal subjects providing a clear indication that SCN synchronizes rhythms in the peripheral tissues. Thus, based on these and many other observations it was concluded that circadian oscillators in the SCN is entrained by environmental light/dark cycles, which in turn synchronizes the peripheral oscillators to time various behaviours and physiological functions.

Light entrainable peripheral oscillators time various behaviours and physiological functions.

In contrast to hierarchical circadian organization observed in mammalian systems, circadian organization in *Drosophila* and zebra fish consist of peripheral oscillators which are directly entrainable to external light/dark cycles. The Plautz *et al* [7] study clearly demonstrated the presence of peripheral oscillators in *Drosophila* and also found that rhythms of *per* expression in isolated tissues were able to re-entrain to new light/dark cycles indicating that peripheral oscillators could sense light and entrain independent of the circadian oscillators in the brain. Another study reported the ability of *per* expression rhythms in the Malpighian tubules to re-entrain to phase-shifted light/dark cycles in decapitated flies thus demonstrating the presence of brain-independent circadian oscillators in *Drosophila*. Similarly, a study by Whitmore *et al* [9] reported that peripheral oscillators in zebra fish are also sensitive to light and can entrain to light/dark cycles without any help from the circadian oscillators in the brain.

The Elusive Circadian Oscillators

Localization of autonomous circadian oscillators in various body tissues and recent studies revealing their organization might give an impression that the mystery of circadian organization has been

resolved. However, several studies in mammals have indicated the presence of circadian timing systems which work independent of the known circadian oscillators. Methamphetamine-sensitive circadian oscillator (MASCO) is one such example. A study on rats in 1987 by Japanese chronobiologist Kenichi Honma and colleagues found that chronic administration of psycho-stimulant drug methamphetamine to SCN lesioned rats can induce robust locomotor activity rhythm under constant conditions (Honma *et al* [12]), indicating that a separate mechanism underlies methamphetamine-induced rhythms in rats. Several studies have even found induction of circadian rhythms in body temperature, feeding, drinking and corticosterone upon chronic administration of methamphetamine. Further studies have shown that methamphetamine also induces rhythms in clock mutant animals. This evidence suggests that methamphetamine induced rhythms or the underlying putative oscillator (MASCO) is independent of SCN and does not involve already known clock mechanisms.

Animals show food anticipatory activity (FAA) when they are given food at a specific time every day and this rhythm shows several properties that indicates its circadian regulation. FAA, in animals entrained to a feeding cycles show re-entrainment with transient cycles after phase-shifted feeding cycles *Figure* 6A. FAA is known to disappear when food is given *ad libitum*; however, FAA reappears during food deprivation following a stretch of *ad libitum* food, and its timing is predictable from the prior entrainment feeding schedules *Figure* 6B, suggesting that the underlying circadian oscillator was free-running. Interestingly, FAA is known to persist in SCN-lesioned animals subjected to restricted feeding cycles. Thus, although these properties of rhythmic FAA indicate circadian oscillators which have been named as food-entrainable oscillator (FEO), attempts to identify their anatomical location and to unravel the underlying molecular mechanisms have not, thus far, been successful.

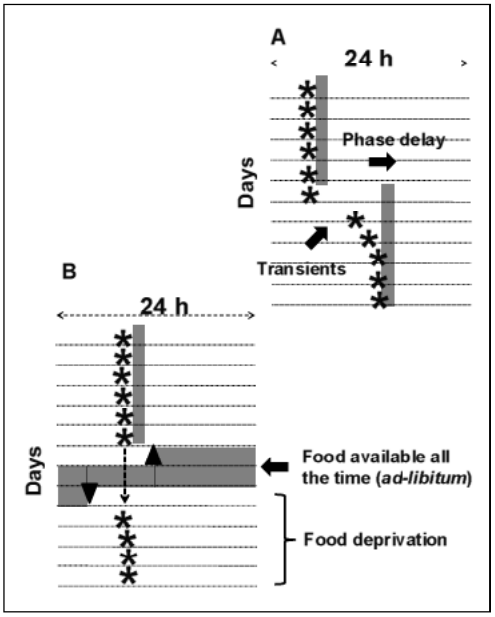

Figure 6. Evidence for food entrainable oscillator.
(FEO). Each horizontal line represents a day. Vertical gray bars represent the time of food availability on each day. Asterisks (*) represents phase of food anticipatory activity (FAA).
(**A**) Re-entrianment to the delayed food availability regime occurs through transients.
(**B**) The reappearance of FAA during food deprivation, following a stretch of *ad libitum* food. The phase of FAA during food deprivation is predictable from the phase of previous food availability schedule.

Conclusion

Observations of internal synchrony among multiple circadian rhythms in individual animal subjects inspired Pittendrigh to propose multi-oscillatory circadian organization. Years of research in animal models ranging from invertebrates to mammals have clearly demonstrated that circadian timing systems are indeed multi-oscillatory. Moreover, further studies have shown that multiple oscillators are organized differently in different animal systems to regulate various behaviours and physiological functions. Methamphetamine-induced rhythms and the rhythm in food anticipatory activity suggests that there is much that is still unexplored in circadian organization.

References

[1] **J Aschoff, Circadian rhythms in man, *Science*, Vol.148, pp.1427–1432, 1965.**

[2] **F M Sulzman, C A Fuller and M C Moore-Ede, Tonic effects of light on the circadian system of the squirrel monkey, *Journal of Comparative Physiology*, Vol.129, pp.43–50, 1979.**

[3] **C S Pittendrigh, Circadian oscillations in cells and the circadian organization of multicellular systems, In *The neurosciences*: *Third study program*, F O Schmitt and F G Worden (Eds.), pp.432–458. MIT Press, Cambridge, 1974.**

[4] **J E Harker, Endocrine and nervous factors in insect circadian rhythms, *Cold Spring Harbour Symposium Quantitative Biology*, Vol.25, pp.279-287, 1960.**

[5] **J Nishiitsutsuji-Uwo and C S Pittendrigh, Central nervous system control of circadian rhythmicity in the cockroach III. The optic lobes, the locus of driving oscillations? *Journal of Comparative Physiology A*, Vol.58, pp.1-13, 1968.**

[6] **T L Page, Transplantation of the cockroach circadian pacemaker, *Science*, Vol.216, pp.73–75, 1982.**

[7] **L D Plautz, M Kanek, J C Hall and S A Kay, Independent photoreceptive circadian clocks throughout *Drosophila*, *Science*, Vol.278, pp.1632–1635, 1997.**

[8] **M J Zylka, L P Shearman, D R Weaver and S M Reppert, Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain, *Neuron*, Vol.20, pp.1103–1110, 1998.**

[9] **D Whitmore, N S Foulkes, U Strahle and P Sassone-Corsi, Zebrafish clock rhythmic expression reveals independent peripheral circadian oscillators, *Nature Neuroscience*, Vol.1, pp.701–707, 1998.**

[10] S Yamazaki, R Numano, M Abe, A Hida, R Takahashi, M Ueda, G D Block, Y Sakaki, M Menaker and H Tei, Resetting central and peripheral circadian oscillators in transgenic rats, *Science*, Vol.288, pp.682–685, 2000.

[11] S H Yoo, S Yamazaki, P L Lowrey, K Shimomura, C H Ko, E D Buhr, S M Siepka, H K Hong, W J Oh, O J Yoo, M Menaker, J S Takahashi, PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues, *Proc. Natl. Acad. Sci. USA*, Vol.101, pp.5339–5346, 2004.

[12] K Honma, S Honma and T Hiroshige, Activity rhythms in the circadian domain appearin suprachiasmatic nuclei lesioned rats given methamphetamine, *Physiology and Behavior*, Vol.40, pp.767–774, 1987.

Suggested Reading

[1] D Bell-Pedersen, V M Cassone, D J Earnest, S S Golden, P E Hardin, T L Thomas and M J Zoran, Circadian rhythms from multiple oscillators: lessons from diverse organisms, *Nat. Rev. Genet.*, Vol.6, pp.544–556, 2005.

[2] S Daan, A history of chronobiological concepts, In *The Circadian Clock*, U Albrecht (Ed.), Springer, Heidelberg, pp.1–36, 2010.

[3] J A Mohawk, C B Green, J S Takahashi, Central and peripheral circadian clocks in mammals, *Annu. Rev. Neurosci.*, Vol.35, pp.445–462, 2012.

[4] M C Moore-Ede, F M Sulzman and C A Fuller, *Organization of circadian timing system*, M C Moore-Ede, F M Sulzman, C A Fuller (Ed.), Harvard University Press, Cambridge, pp.113–151, 2001.

[5] C S Pittendrigh, Circadian rhythms and the circadian organization of living systems, *Cold Spring Harbor Symp. Quant. Biol.*, Vol.25, pp.159–84, 1960.

[6] U Schibler, J Ripperger and S A Brown, Peripheral circadian oscillators in mammals: time and food, *J. Biol. Rhythms*, Vol.18, pp.250–260, 2003.

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