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Peripheral Circadian Oscillators

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Circadian rhythms are ~24-hour cycles of physiology and behavior that are synchronized to environmental cycles, such as the light-dark cycle. During the 20th century, most research focused on establishing the fundamental properties of circadian rhythms and discovering circadian pacemakers that were believed to reside in the nervous system of animals. During this time, studies that suggested the existence of circadian oscillators in peripheral organs in mammals were largely dismissed. The discovery of a single-locus circadian pacemaker in the nervous system of several animals affirmed the single-oscillator model of the circadian system. However, the discovery of the genes that constituted the molecular timekeeping system provided the tools for demonstrating the existence of bona fide circadian oscillators in nearly every peripheral tissue in animals, including rodents, in the late 1990s and early 2000s. These studies led to our current understanding that the circadian system in animals is a hierarchical multi-oscillatory network, composed of master pacemaker(s) in the brain and oscillators in peripheral organs. Further studies showed that altering the temporal relationship between these oscillators by simulating jet-lag and metabolic challenges in rodents caused adverse physiological outcomes. Herein we review the studies that led to our current understanding of the function and pathology of the hierarchical multi-oscillator circadian system.

INTRODUCTION

Circadian rhythms, which are 24-hour cycles of behavior and physiology (*e.g.* sleep/wake and core body temperature cycles), are ubiquitous and observed in most organisms. Before the 1960s, scientists established the three fundamental properties of circadian rhythms. First, the circadian rhythm must be self-sustained and free-run with a period of ~24 hours (*i.e.* the 24h rhythm persists in constant conditions). Second, the circadian rhythm should entrain to (be synchronized by) environmental cycles, such as the light-dark cycle. Third, the circadian rhythm should maintain a ~24h period across a physiological range of temperatures (*i.e.* it is temperature-compensated). After these formal properties were established, the next goal was to discover the locus of the self-sustained circadian oscillator.

During the 1960s and 1970s, scientists searched for the location of *the* circadian pacemaker in multicellular

organisms. At this time, it was believed that a pacemaker(s) located in the brain or in a neuroendocrine gland drives overt circadian rhythms in behavior and physiology. The approaches used were to lesion, transplant, and culture candidate pacemaker tissues. For an organ to qualify as the circadian pacemaker, it had to adhere to the fundamental circadian properties. Lesioning should abolish the overt circadian rhythms and transplantation of that tissue should restore the circadian rhythms. Moreover, the tissue must express a circadian rhythm *in vitro* in constant conditions. Using these methods, the locus of the circadian pacemaker was identified in the central nervous system of several animals—the optic lobe in cockroaches (lesion [1], transplant [2], *in vitro* [3]), the pineal gland in house sparrows (lesion [4], transplant [5,6]) and the suprachiasmatic nucleus (SCN†) of the hypothalamus in rodents (lesion [7,8], *in vivo* isolation [9], transplant [10-13], *in vitro* [14-16]). It was later shown that the pacemakers in non-mammalian vertebrate species are distributed in a circadian axis and include the retina, SCN, and pineal gland. The dominant pacemaker in the axis varies in each species [17-19].

Although the single-pacemaker model of the circadian system was the prevailing view in the 20th century, scientists were also making discoveries suggesting that circadian oscillators could be located in peripheral tissues. This hierarchical multi-oscillator view of the circadian system was not widely accepted. More than 40 years after the initial discoveries that implicated peripheral tissues as circadian clocks, it is now dogma in the field of chronobiology that the circadian system is a hierarchical multi-oscillator network of circadian clocks. Herein we review the studies that led to our current understanding of the hierarchical multi-oscillator circadian system.

EARLIEST STUDIES OF PERIPHERAL CIRCADIAN RHYTHMS: FIRST EVIDENCE OF MULTI-OSCILLATOR CIRCADIAN SYSTEMS

In 1958, Erwin Bünning reported that intestines cultured from golden hamsters expressed ~24h rhythms in peristalsis under a range of temperatures (20-39°C) [20]. After Bünning's report, several studies by G. Edgar Folk and others in the 1960s and 1970s showed that cultured mammalian tissues (adrenal, heart, and liver) exhibited circadian rhythms in metabolism, hormonal secretion, or enzyme activity [21-29]. These studies were published when mammalian circadian biologists were trying to identify a solitary circadian pacemaker in the central nervous system. Therefore, in the field of mammalian circadian rhythms, the existence of circadian oscillators in peripheral organs was not widely acknowledged. In contrast, chronobiologists studying insects had already developed a multi-oscillator model of the insect circadian system after an elegant study by Jaga Giebultowicz and her colleagues showed that the isolated testis-seminal ducts complex from gypsy moths contained a functional self-sustained circadian oscillator that was entrained by light [30].

THE MOLECULAR BIOLOGY ERA: USING REPORTER TECHNOLOGY TO DEMONSTRATE SELF-SUSTAINED CIRCADIAN RHYTHMS IN PERIPHERAL ORGANS

Successful cloning of circadian genes in *Drosophila*, zebrafish, and mammals in the 1980s and 1990s provided the tools to observe molecular rhythms in tissues outside of the central nervous system. Giebultowicz and Hege observed ~24h cycles of expression of the circadian proteins, PERIOD and TIMELESS, in Malpighian tubules in headless *Drosophila* housed in constant dark or entrained to the light-dark cycle [31,32]. Steve Kay and colleagues generated transgenic *Drosophila* in which the promotor of the *period* gene drove firefly luciferase reporter gene expression. Using these transgenic flies, they measured circadian gene transcription from living flies [33], and also measured light emission from cultured tissues [34]. Surprisingly, nearly every tissue, including the antenna, proboscis, wing, and leg, exhibited self-sustained circadian rhythms that entrained to environmental light-dark cycles.

In vertebrates, Tosini and Menaker found that cultured neural retinas from golden hamsters exhibited a circadian rhythm of melatonin release that entrained to light [35]. One year later, in 1997, two mammalian genes, *Clock* and *Period*, were cloned by forward and reverse genetics, respectively [36-39]. Both *Clock* and *Period* were expressed in the central nervous system and in many other peripheral organs in mice and humans. Schibler and colleagues also found that *Period2* and other circadian genes cycled in an immortalized rat fibroblast cell line [40]. At the same time, Sassone-Corsi and colleagues discovered that organs cultured from zebrafish showed rhythmic expression of circadian genes, and later those rhythms were shown to directly entrain to environmental light-dark cycles [41,42].

In 2000, our group was the first to demonstrate self-sustained circadian gene expression in cultured peripheral tissues in mammals [43]. We generated a transgenic rat which carried the *Period1-luciferase* transgene, in which the *Period1* promotor controlled the expression of luciferase. Consistent with the original observation that *Period1* mRNA was expressed in many peripheral organs, tail snips submerged in luciferin were bioluminescent. The first tissues we attempted to culture were the
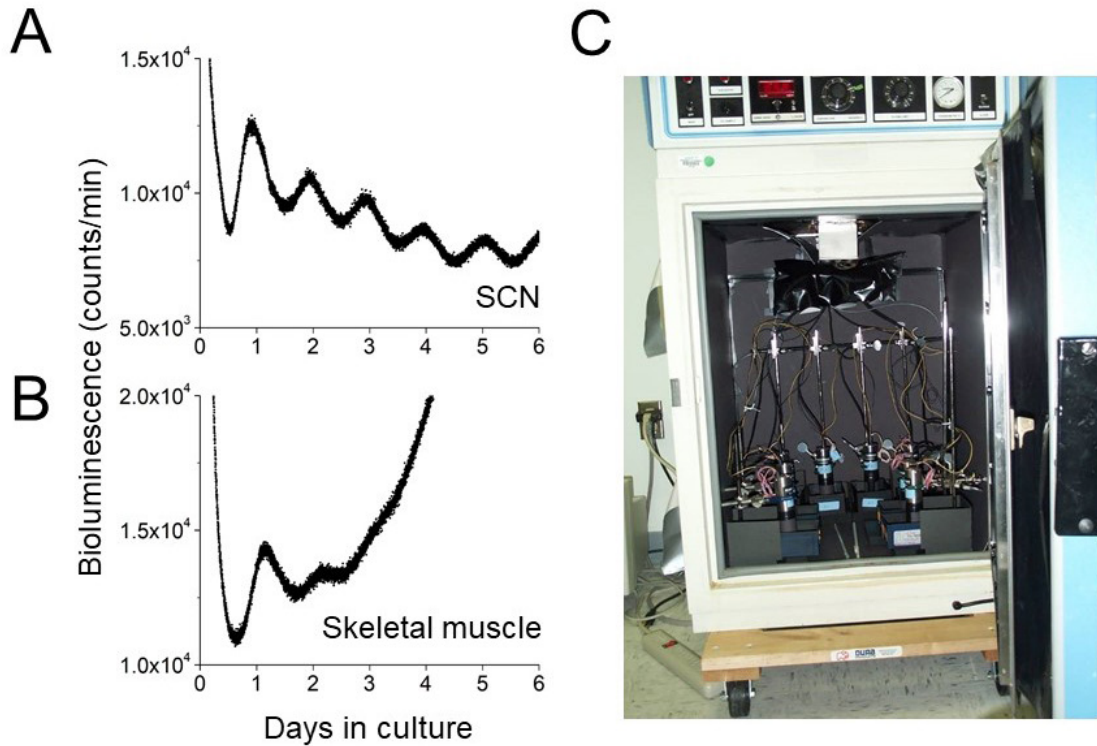
Figure 1. First successful recording of a circadian promoter-driven luminescence rhythm in cultured rodent peripheral tissue. On May 19, 1999, the SCN and skeletal muscle were explanted from a 15 day-old *Period1-luciferase* rat (L1-line) and cultured with 0.1 mM luciferin. This was our second attempt to record the luminescence rhythm. Bioluminescence was continuously monitored from the SCN (A) and muscle (B) by photo-multiplier tubes (HC135, Hamamatsu) maintained in the incubator at 36°C. Photon counts were recorded at 1-min intervals. C: Photo of the original set-up for bioluminescence recording. It had only two photo-multiplier tubes that were extended to 8 channels.

SCN and muscle. We found that cultured muscle exhibited two cycles of a circadian rhythm of bioluminescence (the first *Period1*-bioluminescence recording from cultured muscle is shown in Figure 1B). As expected based on lesion and transplant studies, the *Period1*-bioluminesence circadian rhythm was robust in cultured SCN explants (Figure 1A). In addition, most tissues we cultured also exhibited circadian rhythms. This study transformed our understanding of the mammalian circadian system and demonstrated that it is composed of multiple circadian oscillators, similar to *Drosophila* and zebrafish. In contrast to *Drosophila* and zebrafish, mammalian peripheral oscillators are not light-sensitive and only tissues in the eye (*e.g.* retina, cornea, retinal pigment epithelium-choroid) have been shown to entrain to light-dark cycles *in vitro* [44-48].

To investigate how the hierarchical multi-oscillatory mammalian circadian system entrained to the environmental light-dark cycle, we subjected *Period1-luciferase* transgenic rats to a jet-lag protocol (shifting the time of lights-on 6h earlier to simulate eastward travel or 6h later to simulate westward travel). We found that the SCN circadian rhythm adapted to the new light-dark cycle quickly, but it took several days for peripheral tissue rhythms to entrain to the new light-dark cycle [43]. Importantly, the speed of entrainment was different in each peripheral organ.

Because food availability is a cyclic environmental factor, we next fed *Period1-luciferase* rats only during the daytime. In the presence of the light-dark cycle, rats were given access to food for only 4h during the light phase. Since rats are nocturnal and normally eat during the night, the daytime restricted feeding provided two conflicting environmental cues, light and food. We found that the SCN rhythm entrained to the light-dark cycle (and was unaffected by restricted feeding), while the liver rhythm entrained to feeding time [49]. Two other groups independently observed the same phenomenon using conventional mRNA measurements [50,51].

EMERGENCE OF THE CIRCADIAN MISALIGNMENT CONCEPT

The discovery of peripheral circadian oscillators in mammals was paradigm-shifting; we came to view the mammalian circadian system as a hierarchical multi-oscillatory system rather than a system controlled by one pacemaker structure in the SCN. This new paradigm afforded a series of experiments that measured how development, aging, and metabolic challenges (*e.g.* high-fat diet, exercise) altered the rhythms in peripheral tissues [52-55]. Numerous studies also described the adverse physiological consequences of disruption of the multi-oscillator circadian system. A striking consequence of this disruption was our study that showed aged mice and rats in the jet-lag protocol had increased mortality [56,57]. We found that only 47 percent of aged mice survived repeated 6-h advances of the light-dark cycle, compared to 83 percent survival of aged mice in a typical static light-dark cycle. Circadian misalignment also adversely affects physiology in humans. For example, healthy adult subjects who were forced to sleep and eat on a 28-h cycle became prediabetic when their circadian rhythms were misaligned with the environmental cycle [58].

As the concept of "circadian misalignment" has gained momentum, so has the complexity and diversity of the definitions and experimental paradigms in investigating this concept. For example, circadian misalignment can be internal (*e.g.* desynchrony among peripheral oscillators or among central and peripheral oscillators) or external (*e.g.* the light-dark cycle is not aligned with the internal rhythm), or a combination of these factors (as seen during jet-lag). There can even be misalignment within a pacemaker structure. For example, groups of cellular oscillators within the SCN (*e.g.* the right and left SCN or the ventral and dorsal SCN) can dissociate under certain environmental conditions [59,60].

FUNCTIONAL SIGNIFICANCE OF PERIPHERAL OSCILLATORS

After the new hierarchical multi-oscillator model of the circadian system was established, the next obvious question became: what is the role of a peripheral circadian oscillator? In gypsy moths, it was shown that the circadian oscillator in the testis-seminal ducts complex controlled sperm release [30]. This question was addressed in mammals by generating tissue/cell type-specific circadian gene knockout animals. The *Period* and other circadian genes have multiple paralogs and single gene knockout does not cause arrhythmicity. *Bmal1* is the only single-gene knockout that disabled the circadian oscillator. As a result, most studies have used Cre-lox technology to knock out *Bmal1* and make clock-less tissues. But, somewhat surprisingly, a significant portion of genes, including some circadian genes, continued to cycle in *Bmal1* knockout (or knock down) tissues *in vivo*, because systemic circadian hormonal and physiological signals drove rhythmicity in tissues [61]. Regardless, most of the studies summarized in Table 1 support the hypothesis that circadian oscillators in peripheral tissues control local physiology. For instance, the ERG b-wave rhythm was lost in retina-specific *Bmal1*-knockout mice [62]. Metabolic defects were found in the mice in which *Bmal1* was knocked out in tissues related to metabolism (liver [63], skeletal muscle [64], pancreas [65,66]). Probably the most severe phenotype in tissue-specific *Bmal1* knockouts is shortened life span in cardiomyocyte-specific knockouts [67]. Knocking out *Bmal1* in ovarian steroidogenic cells or theca cells decreased fertility and litter size [68,69]. An interesting finding in tissue-specific knockouts is that the effects of disabling the clock in a tissue can extend beyond the function of that tissue. Paul and colleagues found changes in the total amount of non-REM sleep in the mouse when *Bmal1* was knocked out in muscle [70]. This could be due, in part, to the heterogeneous functions of BMAL1 both in the output of the circadian oscillator and the non-circadian roles of BMAL1. *Bmal1* has a paralog, *Bmal2*, which is down-regulated in *Bmal1*-knockout tissues [71]. CLOCK/NPAS2 and BMAL1/BMAL2 are transcription factors that activate thousands of E-box-containing genes. Therefore, knocking out *Bmal1* in a tissue not only disables the circadian oscillator, but also causes an array of other genes to be aberrantly regulated. Therefore, the tissue-specific functions of peripheral clocks must be confirmed by knocking out other circadian genes that are not transcription activators (*e.g. Period1/2* or *Cryptochrome1/2* double knockouts).

HIERARCHICAL ORGANIZATION OF THE MULTI-OSCILLATOR SYSTEM: CIRCADIAN PACEMAKER(S) AT THE TOP OF THE HIERARCHY

The central circadian pacemaker, the SCN, is necessary and sufficient for circadian rhythms in behavior and physiology. To understand the relationship between the SCN and peripheral oscillators, the function of the SCN was disabled by either lesion or by *Bmal1* knockout in the brain (this knockout included, but was not exclusive to, the SCN). These studies support the hypothesis that the SCN coordinates the phases of peripheral oscillators. Peripheral clocks remained rhythmic in both SCN-lesioned and brain-*Bmal1* knockout mice, but the phase relationship between peripheral oscillators was disrupted [72-74]. Together, many studies have contributed to the metaphor of the mammalian circadian system as a sym-

Table 1. Physiological Consequences of Tissue-Specific *Bmal1* Deletion.

Tissue Bmal1 deleted / Cre Driver	Key Results	References
Retina / CHX10-Cre	ERG b-wave rhythm was lost	Storch et al. (2007) [62]
Liver / Albumin-Cre	Hypoglycemia during fasting phase	Lamia et al. (2008) [63]
Liver / Albumin-Cre	Increased expression of lipoprotein lipase mRNA	Shimba et al. (2011) [81]
Pancreatic islet / PDX1-Cre	Impaired glucose tolerance / hyperglycemia	Marcheva et al. (2010) [65]
Pancreatic islet / PDX-CreER*	Impaired glucose tolerance / hyperglycemia / hypoinsulinemia	Perelis et al. (2015) [66]
Adipocyte / adipocyte protein 2-Cre or adiponectin-Cre	Obese / reduced amplitude of food intake rhythm / reduced energy expenditure	Paschos et al. (2012) [82]
Skeletal muscle / muscle creatine kinase-Cre	No phenotype	Shimba et al. (2011) [81]
Skeletal muscle / human skeletal actin-MerCreMer*	Disrupted glucose metabolism / hyperglycemia in non-fasting / glucose intolerance / altered body composition / increased amount of non-REM sleep	Hodge et al. (2015) [83] Harfmann et al. (2016) [64] Ehlen et al. (2017) [70]
Cardiomyocyte / αMHC-Cre	Shortened life span / accelerated age-dependent-dilated cardiomyopathy	Young et al. (2014) [67] Ingle et al. (2015) [84]
Smooth muscle / SM22α-Cre	Reduced amplitude blood pressure rhythms	Xie et al. (2015) [85]
Perivascular adipose tissue (Brown adipocyte) / UCP1-Cre	Reduced blood pressure during resting phase	Chang et al. (2018) [86]
Adrenal / MC2R[#]	No alteration corticosterone rhythm under light-dark cycle, but amplitude of rhythm is diminished under constant darkness	Son et al. (2008) [87]
Adrenal / aldosterone synthase-Cre	No alteration in corticosterone rhythm under regular light-dark cycle (12:12)	Engeland et al., (2018) [88]
Renal tubular cell / Pax8-rtTA/LC1[φ]	Small kidney size / increased plasma urea level	Nikolaeva et al. (2016) [89]
Ovarian steroidogenic cell / SF1-Cre	Impaired uterine implantation / worsened fertility	Liu et al. (2014) [68]
Ovarian theca cell / Cyp17-Cre	Abolished daily rhythm of oocyte release in response to eLH / small litter size (subfertile)	Mereness et al. (2016) [69]
Ovarian granulosa cell / Cyp19-Cre	No abnormality was observed	Mereness et al. (2016) [69]
Pituitary gonadotrope cell / GnRHR-internal ribosome entry site-Cre	Increased estrous cycle length variability / no changes in litter size	Chu et al. (2013) [90]
Myeloid / LysM-Cre	Increased size of atherosclerotic lesion in Apoe-/- background	Huo et al. (2017) [91]

*tamoxifen inducible; [#]knockdown by *Bmal1* antisense; [φ]doxycycline inducible

phony, where the SCN is the conductor and the peripheral oscillators are the musicians (Figure 2) [75].

The SCN is not the only circadian pacemaker that is capable of acting as the conductor of the symphony. It is known that at least two SCN-independent circadian pacemakers exist in rodents [76,77]. One is the food-entrainable oscillator (FEO), which controls food anticipatory activity during time-restricted feeding. The second is the methamphetamine-sensitive circadian oscillator (MASCO), whose behavior rhythm (MASCO-driven activity rhythm) appears when low-dose methamphetamine is chronically administered to rodents. Interestingly, both the FEO and MASCO do not depend on canonical circadian genes to keep time [76,77]. By measuring the phases of luminescence rhythms from *ex vivo* tissues, it was shown that both the FEO and MASCO can substitute
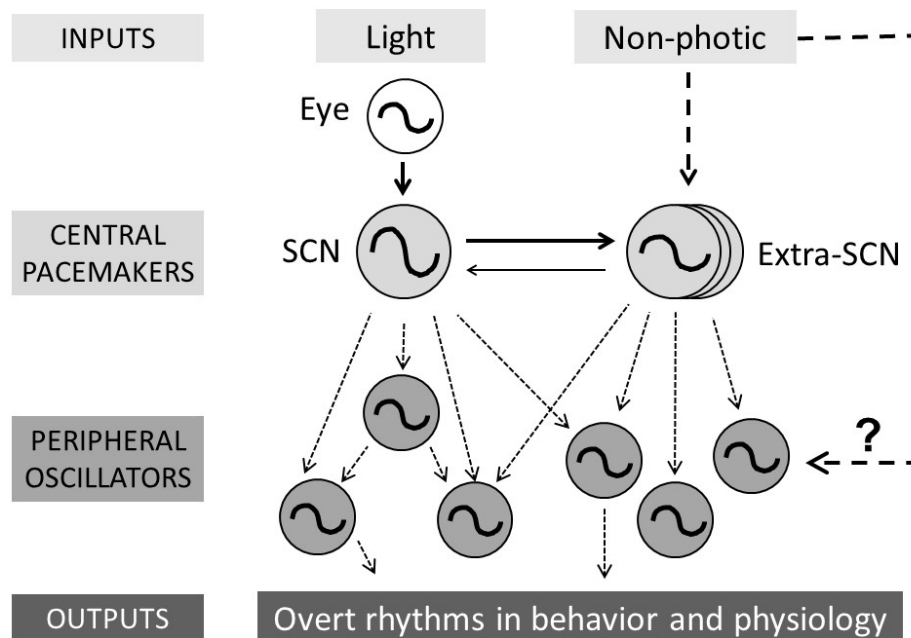
Figure 2. Current working model of the hierarchical multi-oscillatory mammalian circadian system. Light (via the eye) entrains the SCN and non-photic inputs (*e.g.* food, palatable meal, wheel-running) entrain (via unknown pathways) extra-SCN pacemakers. The SCN and extra-SCN pacemakers coordinate the phases of peripheral oscillators. The output pathways that control overt rhythms of behavior and physiology are largely unknown. Dotted lines represent unknown pathways.

for the SCN and coordinate the phases of peripheral oscillators when the SCN is lesioned or the circadian clock in the SCN is disabled [73,78]. However, the anatomical loci and the roles of the extra-SCN pacemakers under normal conditions (*i.e.* without restricted feeding and without methamphetamine), when the SCN is present and functional, remain to be elucidated.

THE NEXT FRONTIER

Although the hierarchical multi-oscillatory nature of the circadian system is now well established, many questions remain to be answered. We still have much to learn about the physiological significances of peripheral oscillators. Perhaps the most understudied aspect of the mammalian circadian system is the output pathways of the SCN. For example, although we know the SCN is necessary and sufficient for the circadian rhythm of locomotor activity, we know very little about the neural circuitry downstream of the SCN that controls this rhythm. Neuroanatomical studies have shown that the SCN primarily projects to the subparaventricular zone (SPZ) and dorsomedial nucleus of the hypothalamus (DMH). Lesion studies have shown that the SPZ and DMH participate in regulation of circadian rhythms of sleep, locomotor activity, eating, and body temperature, but the specific neural and hormonal output/modulatory pathways remain to be elucidated [79]. In the reciprocal direction, we have shown that the eyes and the SCN are coupled and stabilize the locomotor activity rhythm of the hamster in constant darkness [80]. Understanding the ways that the SCN and peripheral oscillators interact will reveal the network architecture of the circadian system and further elucidate the physiological functions of peripheral oscillators.

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