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Achim Kramer
Martha Merrow *Editors*

Circadian Clocks

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Circadian Clocks

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Preface

The human body functions as a 24-h machine: remarkably, this machine keeps going with a *circa* 24-h rhythm in sleeping and waking, in physiologies such as blood pressure and cortisol production, in cognitive functions, and indeed also in expression of circa 10–20 % of the genome in any given cell. The circadian (from the Latin "*circa diem*" or about a day) clock controls all of these processes with a molecular mechanism that is pervasive, as we now know that essentially every cell of our body is oscillating. Furthermore, our cells apparently utilize a circadian clock mechanism with a similar molecular makeup. The recent years have witnessed an enormous progress in our understanding of the mechanistic and genetic basis of this regulation, which we have tried to highlight in this volume.

The circadian clock is relevant for heath—clock gene mutants show reduced fitness, increased cancer susceptibility and metabolic diseases. In addition, drug efficacy and toxicity often vary with time of day with huge implications for therapeutic strategies. The intention of this book is to provide the reader with a comprehensive and contemporary overview about the molecular, cellular and system-wide principles of circadian clock regulation. In keeping with the focus of the *Handbook of Experimental Pharmacology* series, emphasis is placed on methods as well as the importance of circadian clocks for the timing of therapeutic interventions. Despite the decades-old practice of administration of cortisol on the morning, chronopharmacology and chronotherapy are still mostly at an experimental level. Thus, knowledge about the widespread impact of circadian clocks should be invaluable for a broad readership not only in basic science but also in translational and clinical medicine.

This book contains four topical sections. Part I is devoted to describing our current knowledge about the molecular and cellular bases of circadian clocks. In the first chapter, the readers learn about clock genes and the intracellular genetic network that generates ~24-h rhythms on the molecular level. The second chapter focuses on how the circadian clock is using epigenetic mechanisms to regulate the circadian expression of as many as 10 % of cellular transcripts. The following two chapters focus on the hierarchy of mammalian circadian organization: the clock in the brain is the master pacemaker, often controlling daily timing in peripheral

tissues. The mechanisms of these synchronization processes within tissues and organisms are discussed.

Part II of the book is devoted to describing how and what is controlled by the circadian clock. The general term for this is *outputs* of the clock. Here, we will cover sleep, metabolism, hormone levels and mood-related behaviors that are especially relevant to pharmacology. In recent years, the reciprocal control of metabolic processes and the circadian system emerged, which is the focus of the first chapter of this part. This connection has been elucidated both on a molecular basis and also in epidemiological studies. Several common themes will emerge including the feedbacks between clocks and the clock output systems as well as the balance between local and tissue-specific clocks and the system-wide control of circadian functions. Concerning human behavior, there is nothing more disparate than the states of sleep and wakefulness; the reader will learn that the timing of these states is profoundly governed by the circadian clocks and its associated genes (see also Part III, Roenneberg et al.). Single point mutations in clock genes can dramatically alter sleep behavior. Disruption of temporal organization—clock gene mutations or shift work—can lead to health problems and behavioral disorders related to mood alterations. The last chapter in this section discusses these connections and possible *pharmacological* interventions such as light or lithium therapy.

The aim of Part III is to discuss the implications of a circadian system for pharmacology. The first chapter reviews studies from the past several decades that describe daily changes in drug absorption, distribution, metabolism, and excretion. In addition, drug efficacy is controlled by the circadian system due to daily changes in the levels and functionality of many drug targets. The second chapter exemplifies these principles for anticancer therapy, where chronotherapy is relatively advanced. This may be based on the fact that cancer cells have less synchronized circadian clocks. Modulating or strengthening the molecular clock by pharmacological intervention is a strategy that is addressed in one of the contributions in this section. High-throughput screening approaches for small molecules that are capable of pharmacological modulation of the molecular clock are described—this may develop into a valuable approach for both scientific and therapeutic purposes. The last chapter in this section focuses on the role of light for the synchronization of the human clock to our environment (entrainment). Light is the primary synchronizer (*zeitgeber*), and novel light-sensitive cells in the retina mediate entrainment, which is conceptually and epidemiologically analyzed. In shift work, as well as in everyday working life, the dissociation of internal and external time leads to health problems, suggesting the need for intervention strategies that use light as though it were a prescription drug.

Finally, Part IV of this book is devoted to systems biology approaches to our understanding of circadian clocks. In general, our field has relied on models to enhance our conceptual understanding of the highly complex circadian system. The iterative approach of improving models with data from high throughput approaches and feeding back the results for experiments suggested therein—in essence, modern systems biology—is developing into a major tool in our chronobiology repertoire.

In the first chapter of this section, the principles of rhythm generation will be described from a mathematical perspective. It will become clear that feedback loops and coupling are fundamental concepts of oscillating systems. How these fundamentals are used to create rhythms that regulate, for example, transcription at many different times of day is highlighted in the second chapter of this part. The last chapters again help to appreciate the pervasiveness of circadian regulation by focusing on genome- and proteome-wide studies that uncovered circadian rhythms almost everywhere.

This volume adds up to an up-to-date review on the state of chronobiology, particularly with respect to molecular processes. It should be of special interest to chronobiologists, pharmacologists, and any scientists who is concerned with excellent protocols and methods.

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Part I
Molecular and Cellular Basis of Circadian Clocks

Molecular Components of the Mammalian Circadian Clock

Ethan D. Buhr and Joseph S. Takahashi

Abstract Mammals synchronize their circadian activity primarily to the cycles of light and darkness in the environment. This is achieved by ocular photoreception relaying signals to the suprachiasmatic nucleus (SCN) in the hypothalamus. Signals from the SCN cause the synchronization of independent circadian clocks throughout the body to appropriate phases. Signals that can entrain these peripheral clocks include humoral signals, metabolic factors, and body temperature. At the level of individual tissues, thousands of genes are brought to unique phases through the actions of a local transcription/translation-based feedback oscillator and systemic cues. In this molecular clock, the proteins CLOCK and BMAL1 cause the transcription of genes which ultimately feedback and inhibit CLOCK and BMAL1 transcriptional activity. Finally, there are also other molecular circadian oscillators which can act independently of the transcription-based clock in all species which have been tested.

Keywords Circadian • Clock • Molecular

1 Introduction

As the sun sets, nocturnal rodents begin to forage, nocturnal birds of prey begin their hunt while diurnal birds of prey sleep, filamentous fungi begin their daily production of spores, and cyanobacteria begin nitrogen fixation in an environment

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of low O_2 after the day's photosynthesis. As the sun rises the next morning, many plants have positioned their leaves to catch the first rays of light, and many humans sit motionless in cars on a nearby gridlocked highway. It is now understood that the obedience to temporal niches in these and all organisms is governed by a molecular circadian clock. These clocks are not driven by sunlight but are rather synchronized by the 24-h patterns of light and temperature produced by the earth's rotation. The term circadian is derived from "circa" which means "approximately" and "dies" which means "day." A fundamental feature of all circadian rhythms is their persistence in the absence of any environmental cues. This ability of clocks to "free-run" in constant conditions at periods slightly different than 24 h, but yet synchronize, or "entrain," to certain cyclic environmental factors allows organisms to anticipate cyclic changes in the environment. Another fundamental feature of circadian clocks is the ability to be buffered against inappropriate signals and to be persistent under stable ambient conditions. This robust nature of biological clocks is well illustrated in the temperature compensation observed in all molecular and behavioral circadian rhythms. Here temperature compensation refers to the rate of the clock being nearly constant at any stable temperature which is physiologically permissive. The significance of temperature compensation is especially evident in poikilothermic animals that contain clocks that need to maintain 24-h rhythmicity in a wide range of temperatures. Combined, the robust oscillations of the molecular clocks (running at slightly different rates in different organisms) and their unique susceptibility to specific environmental oscillations contribute to and fine-tune the wide diversity of temporal niches observed in nature.

However, the circadian clock governs rhythmicity within an organism far beyond the sleep: activity cycle. In humans and most mammals, there are ~24-h rhythms in body temperature, blood pressure, circulating hormones, metabolism, retinal electroretinogram (ERG) responses, as well as a host of other physiological parameters (Aschoff 1983; Green et al. 2008; Cameron et al. 2008; Eckel-Mahan and Storm 2009). Importantly, these rhythms persist in the absence of light–dark cycles and in many cases in the absence of sleep–wake cycles. On the other side of the coin, a number of human diseases display a circadian component, and in some cases, human disorders and diseases have been shown to occur as a consequence of faulty circadian clocks. This is evident in sleep disorders such as delayed sleep phase syndrome (DSPS) and advanced sleep phase syndrome (ASPS) in which insomnia or hypersomnia result from a misalignment of one's internal time and desired sleep schedule (Reid and Zee 2009). In familial ASPS (FASPS), the disorder cosegregates both with a mutation in the core circadian clock gene *PER2* and independently with a mutation in the PER2-phosphorylating kinase, CK1 δ (Toh et al. 2001; Xu et al. 2005). Intriguingly, transgenic mice engineered to carry the same single amino acid change in PER2 observed in FASPS patients recapitulate the human symptoms of a shortened period (Xu et al. 2007). Although these mutations are likely not the end of the story for these disorders, they give insight into the way molecular clocks affect human well-being. Jet lag and shift work sleep disorder are other examples of health issues where the internal circadian clock is desynchronized from the environmental rhythms. In addition to sleep-related

disorders, circadian clocks are also directly linked with feeding and cellular metabolism, and a number of metabolic complications may result from miscommunication with the circadian clock and metabolic pathways (Green et al. 2008). For example, loss of function of the clock gene, *Bmal1*, in pancreatic beta cells can lead to hypoinsulinemia and diabetes (Marcheva et al. 2010). Finally, some health conditions show evidence of influence of the circadian clock or a circadian clock-controlled process. For example, myocardial infarction and asthma episodes show strong nocturnal or early morning incidence (Muller et al. 1985; Stephenson 2007). Also, susceptibility to UV light-induced skin cancer and chemotherapy treatments varies greatly across the circadian cycle in mice (Gaddameedhi et al. 2011; Gorbacheva et al. 2005).

In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus is the master circadian clock for the entire body (Stephan and Zucker 1972; Moore and Eichler 1972; Slat et al. 2013). However, the SCN is more accurately described as a "master synchronizer" than a strict pacemaker. Most tissues and cell types have been found to display circadian patterns of gene expression when isolated from the SCN (Balsalobre et al. 1998; Tosini and Menaker 1996; Yamazaki et al. 2000; Abe et al. 2002; Brown and Azzi 2013). Therefore, the SCN serves to synchronize the individual cells of the body to a uniform internal time more like the conductor of an orchestra rather than the generator of the tempo themselves. The mammalian SCN is entrained to light cycles in the environment by photoreceptors found exclusively in the eyes (Nelson and Zucker 1981). The SCN then relays phase information to the rest of the brain and body via a combination of neural, humoral, and systemic signals which will be discussed in more detail later. Light information influencing the SCN's phase, the molecular clock within the SCN, and the SCN's ability to set the phase of behavior and physiology throughout the body constitute the three necessary components for a circadian system to be beneficial to an organism (1) environmental input, (2) a self-sustained oscillator, and (3) an output mechanism.

2 Mechanism of the Molecular Circadian Clock

2.1 Transcriptional Feedback Circuits

The molecular clock mechanism in mammals is currently understood as a transcriptional feedback loop involving at least ten genes (Fig. 1). The genes *Clock* and *Bmal1* (or *Mop3*) encode bHLH-PAS (basic helix–loop–helix; *Per-Arnt-Single-minded*, named after proteins in which the domains were first characterized) proteins that form the positive limb of the feedback circuit [reviewed in Lowrey and Takahashi (2011)]. The CLOCK/BMAL1 heterodimer initiates the transcription by binding to specific DNA elements, E-boxes (5′-CACGTG-3′), and E′-boxes (5′-CACGTT-3′) in the promoters of target genes (Gekakis et al. 1998; Yoo et al. 2005; Ohno et al. 2007). This set of activated genes includes members of the

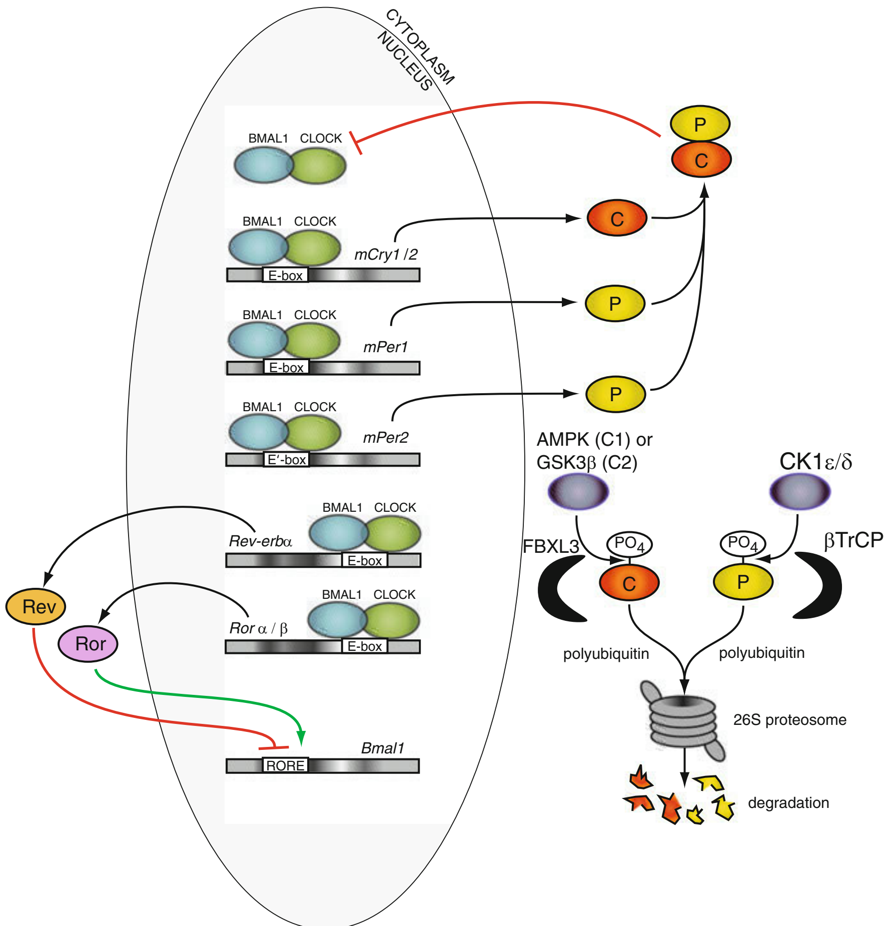

Fig. 1 Schematic of the molecular clock of mammals. CLOCK/BMAL1 heterodimers (*green* and *blue ovals*) bind DNA of clock target genes at E-boxes or E′-boxes and initiate the transcription of their RNA. The resulting PER and CRY proteins (*red* and *yellow ovals*) dimerize in the cytoplasm and translocate to the nucleus where they inhibit CLOCK/BMAL1 proteins from initiating further transcription

negative limb of the feedback loop including the *Per* (*Per1 and Per2*) and *Cry* (*Cry1* and *Cry2*) genes (Gekakis et al. 1998; Hogenesch et al. 1998; Kume et al. 1999). The resulting PER and CRY proteins dimerize and inhibit further CLOCK/BMAL1 transcriptional activity, allowing the cycle to repeat from a level of low transcriptional activity (Griffin et al. 1999; Sangoram et al. 1998; Field et al. 2000; Sato et al. 2006). The chromatin remodeling necessary for this cyclic transcriptional activity is achieved by a combination of clock-specific and ubiquitous histone-modifying proteins and can be observed in the rhythmic acetylation/deacetylation of histones (H3 and H4) at multiple clock target genes (Etchegaray et al. 2003; Ripperger and Schibler 2006; Sahar and Sassone-Corsi 2013). The CLOCK protein

itself possesses a histone acetyl transferase (HAT) domain which is necessary for the rescue of rhythms in *Clock*-mutant fibroblasts (Doi et al. 2006). The CLOCK/BMAL1 complex also recruits the methyltransferase MLL1 to cyclically methylated histone H3 and HDAC inhibitor JARID1a to further facilitate transcriptional activation (Katada and Sassone-Corsi 2010; DiTacchio et al. 2011). Deacetylation takes place, in part, due to recruitment by PER1 of the SIN3-HDAC (SIN3-histone deacetylase) complex to CLOCK/BMAL1-bound DNA, and more members of the circadian deacetylation process are sure to be elucidated (Duong et al. 2011). Intriguingly, the rhythmic deacetylation of histone H3 at the promoters of circadian genes is regulated by the deacetylase SIRT1, which is sensitive to NAD^+ levels (Nakahata et al. 2008; Asher et al. 2008). This is interesting considering that the NAD^+ to NADH ratio has been shown to regulate CLOCK/BMAL1's ability to bind DNA in vitro (Rutter et al. 2001). Thus, cellular metabolism may prove to play an important role in regulating the transcriptional state, and therefore the phase, of the clock (see also Marcheva et al. 2013).

Degradation of the negative limb proteins PER and CRY is required to terminate the repression phase and restart a new cycle of transcription. The stability/degradation rate of the PER and CRY proteins is key to setting the period of the clock. The first mammal identified as a circadian mutant was the *tau*-mutant hamster which displays a free-running period of 20 h, compared to a wild-type free-running period of 24 h (Ralph and Menaker 1988). This shortened period results from a mutation in the enzyme casein kinase 1ε (CK1ε), a kinase which phosphorylates the PER proteins (Lowrey et al. 2000). Another casein kinase, CK1δ, was later found to phosphorylate the PER proteins and that this CK1ε/δ-mediated phosphorylation targets the PER proteins for ubiquitination by βTrCP and degradation by the 26S proteasome (Camacho et al. 2001; Eide et al. 2005; Shirogane et al. 2005; Vanselow et al. 2006). Similar to PER, mutant animals with unusual free-running periods (although longer than wild type in these cases) led to elucidation of the degradation pathway of CRY proteins. In two independent examples, a chemically induced mutation responsible for long-period phenotypes in mice was found in the F-box gene *Fbxl3* (Siepka et al. 2007; Godinho et al. 2007). FBXL3 polyubiquitinates CRY proteins, thereby targeting them for proteosomal degradation (Busino et al. 2007). Interestingly, CRY1 and CRY2 are targeted for ubiquitination by unique phosphorylation events and kinases. CRY1 is phosphorylated by AMPK1 and CRY2 by a sequential DYRK1A/GSK-3β cascade (Lamia et al. 2009; Harada et al. 2005; Kurabayashi et al. 2010).

The paralogs of the *Per* genes (*Per1* and *Per2*) and the *Cry* genes (*Cry1* and *Cry2*) have nonredundant roles. Three independent null alleles of *Per1* yielded mice with free-running periods 0.5–1 h shorter than wild types, but a loss of *Per2* produced mice with a 1.5-h period reduction (Zheng et al. 2001; Cermakian et al. 2001; Bae et al. 2001; Zheng et al. 1999). However, the behavior of the *Per2* null mice only remained rhythmic for less than a week before becoming arrhythmic (Bae et al. 2001; Zheng et al. 1999). Knockout alleles of the *Cry* paralogs produced opposite effects. $Cry1^{-/-}$ mice ran 1 h shorter than wild-type mice, while $Cry2^{-/-}$ mice ran 1 h longer (Thresher et al. 1998; Vitaterna et al. 1999; van der Horst et al. 1999).

At the molecular level, further unique properties of the individual paralogs appear, specifically paralog compensation. Paralog compensation means that when one gene of a family is lost or reduced, the expression of a paralog of that gene is increased to partially compensate. A reduction in *Per1* or *Cry1* produced an increase in *Per2* or *Cry2*, respectively (Baggs et al. 2009). However, reductions or loss of *Per2* or *Cry2* did not produce compensatory expression of their respective paralogs (Baggs et al. 2009). Perhaps network features such as these give insight into the differences seen at the behavioral level of the individual null alleles. Importantly, at both the behavioral and molecular level, at least one member of each family is critical for circadian rhythmicity, as $Per1^{-/-};Per2^{-/-}$ mice and $Cry1^{-/-};Cry2^{-/-}$ mice display no signs of intrinsic circadian rhythmicity (Bae et al. 2001; Zheng et al. 1999; Thresher et al. 1998; Vitaterna et al. 1999; van der Horst et al. 1999).

Our laboratory has recently interrogated on a genome-wide level the *cis*-acting regulatory elements (cistrome) of the entire CLOCK/BMAL1 transcriptional feedback loop in the mouse liver (Koike et al. 2012). This has revealed a global circadian regulation of transcription factor occupancy, RNA polymerase II recruitment and initiation, nascent transcription, and chromatin remodeling. We find that the circadian transcriptional cycle of the clock consists of three distinct phases—a poised state, a coordinated de novo transcriptional activation state, and a repressed state. Interestingly only 22 % of mRNA-cycling genes are driven by de novo transcription, suggesting that both transcriptional and posttranscriptional mechanisms underlie the mammalian circadian clock. We also find that circadian modulation of RNAPII recruitment and chromatin remodeling occurs on a genome-wide scale far greater than that seen previously by gene expression profiling (Koike et al. 2012). This reveals both the extensive reach of the circadian clock and potential functions of the clock proteins outside of the clock mechanism.

The members of the negative limb, in particular the PERs, act as the state variable in the mechanism (Edery et al. 1994). Briefly, this means that the levels of these proteins determine the phase of the clock. In the night, when levels of the PER proteins are low, acute administration of light causes an induction in *Per1* and *Per2* transcription (Albrecht et al. 1997; Shearman et al. 1997; Shigeyoshi et al. 1997). With light exposure in the early night, behavioral phase delays are observed, and this corresponds to light-induced increases of both PER1 and PER2 proteins observed in the SCN (Yan and Silver 2004). In the second half of the night, only PER1 levels rise with light exposure, and this corresponds to phases of the night when light-induced phase advances occur (Yan and Silver 2004). These delays in behavior when light is present in the early night and advances in the late night/early morning are sufficient to support entrainment of an animal to a light–dark cycle. If a master clock is running shorter than 24 h, the sensitive delay region of the state variables will receive light and will slightly delay daily, thus tracking dusk. If the clock is running at a period longer than 24 h, the advance region will be affected and cause a daily advance in rhythms, and the animal's behavior will track dawn. The light activation of the *Per* genes is achieved through CREB/MAPK signaling acting on cAMP-response elements (CRE) in the *Per* promoters (Travnickova-Bendova et al. 2002).

The CLOCK/BMAL1 dimers also initiate the transcription of a second feedback loop which acts in coordination with the loop described above. This involves the E-box-mediated transcription of the orphan nuclear-receptor genes *Rev-Erb*α/β and RORα/β (Preitner et al. 2002; Sato et al. 2004; Guillaumond et al. 2005). The REV-ERB and ROR proteins then compete for *r*etinoic acid-related *o*rphan receptor *r*esponse *e*lement (RORE) binding sites within the promoter of *Bmal1* where ROR proteins initiate *Bmal1* transcription and REV-ERB proteins inhibit it (Preitner et al. 2002; Guillaumond et al. 2005). This loop was originally acknowledged as an accessory loop due to the subtle phenotypes observed in mice with individual null alleles of any one of these genes. While a traditional double-knockout is lethal during development, inducible double knockout strategies have allowed the deletion of *Rev-Erbα* and *β* in an adult animal. This has revealed that the *Rev-erb*s are necessary for normal period regulation of circadian behavioral rhythmicity (Cho et al. 2012). A separate set of PAR bZIP genes which contain D-box elements in their promoters make up another potential transcriptional loop. These include genes in the HLF family (Falvey et al. 1995), DBP (Lopez-Molina et al. 1997), TEF (Fonjallaz et al. 1996), and Nfil3 (Mitsui et al. 2001). If one considers just the rate of transcription/translation and the E-box transcription loop described for the *Per*/*Cry* genes alone, it would be easy to imagine the whole cycle taking significantly less than a day or even less than several hours. It has been proposed that the three known binding elements together provide the necessary delay to cycle at near 24 h: E-box in the morning, D-box in the day, and RORE elements in the evening (Ukai-Tadenuma et al. 2011, for a review see Minami et al. 2013). Although no genes, or even gene families, in these D-box accessory loops are required for clock function, they may serve to make the core oscillations more robust and add precision to the period (Preitner et al. 2002; Liu et al. 2008).

2.2 *Non-transcriptional Rhythms*

In some specific examples, the minimum elements required for molecular 24-h rhythms do not include transcription or translation. In the cyanobacterium *Synechococcus*, 24-h rhythms of phosphorylation of the KaiC protein are observed when the proteins KaiA, KaiB, and KaiC are isolated in a test tube in the presence of ATP (Nakajima et al. 2005). The auto-phosphorylation and auto-dephosphorylation of KaiC are mediated by the phosphorylation promoting KaiA and the dephosphorylation promoting KaiB (Iwasaki et al. 2002; Kitayama et al. 2003; Nishiwaki et al. 2000). Later, circadian rhythms which are independent of transcription were discovered in organisms as diverse as algae and humans. In *Ostreococcus tauri* algae, transcription stops in the absence of light; however, the 24-h oxidation cycles of the antioxidant proteins peroxiredoxins continue in constant darkness (O'Neill et al. 2011). Similarly, in human red blood cells, which lack nuclei, peroxiredoxins are oxidized with a circadian rhythm (O'Neill and Reddy 2011). These transcription-lacking oscillators are also temperature compensated and entrainable to temperature

cycles fulfilling other necessary attributes of true circadian clocks (Nakajima et al. 2005; O'Neill and Reddy 2011; Tomita et al. 2005). It should be noted, however, that in nucleated cells the transcriptional clock influences the cytoplasmic peroxiredoxin clock (O'Neill and Reddy 2011). The peroxiredoxin oscillators are remarkably conserved among all phyla that have been examined (Edgar et al. 2012). It is likely that there are more molecular circadian rhythms that can persist without the transcriptional oscillator left to be discovered and that the communication between these and transcriptional molecular clocks will reveal a whole new level of regulation of circadian functions within a single cell (see also O'Neill et al. 2013).

3 Peripheral Clocks

The transcriptional feedback loop described above can be observed not only in the SCN but also in nearly every mammalian tissue (Stratmann and Schibler 2006; Brown and Azzi 2013). If viewed at the single-cell level, the molecular clockwork of transcription and translation can be observed as autonomous single-cell oscillators (Nagoshi et al. 2004; Welsh et al. 2004). In addition to the core clock genes, hundreds or even thousands of genes are expressed with a circadian rhythm in various tissues, but this is not to say there are hundreds of clock genes. Imagine that the core circadian genes act like the gears of a mechanical clock that has hundreds of hands pointing to all different phases but moving at the same rate. Various cellular pathways and gene families pay attention to the hand of the clock in the proper phase for their individual function. It is the same set of core clock components (gears) that drive the phase messengers (hands of the clock) which vary greatly depending on the cell type.

The extent to which the global transcription in a cell was controlled by the circadian clock was not appreciated until the implement of genome-wide tools (Hogenesch and Ueda 2011; Reddy 2013). Between 2 and 10 % of the total genome is transcribed in a circadian manner in various mouse tissues (Kornmann et al. 2001; Akhtar et al. 2002; Panda et al. 2002a; Storch et al. 2002, 2007; Miller et al. 2007). In a study comparing gene expression profiles of ~10,000 genes and expressed sequence tags (EST) in the SCN and liver, 337 genes were found to be cyclic in the SCN and 335 in the liver with an overlap of only 28 genes cycling in both (Panda et al. 2002a). Another study found a similar overlap of only 37 rhythmic genes between the liver and heart while each tissue expressed more than 450 genes (out of 12,488 analyzed) with a circadian rhythm (Storch et al. 2002). The differences in the exact number of genes found to be cycling in a given tissue between studies is almost certainly the result of experimental and analytical variation. Indeed more recent genome-wide transcriptome analyses have revealed many thousands of cycling transcripts in the liver (Hogenesch and Ueda 2011). Circadian gene expression in each tissue is tissue-specific and optimized to best accommodate that tissue's respective function throughout a circadian cycle.

The clock-controlled genes in various tissues are involved in diverse gene pathways depending on the tissue. In the retina, for example, nearly 300 genes show rhythmic expression in darkness, and this includes genes involved in photo-reception, synaptic transmission, and cellular metabolism (Storch et al. 2007). The number of oscillating genes jumps to an astonishing ~2,600 genes in the presence of a light–dark cycle, and these are phased around the cycle suggesting they are not merely driven by the light. Importantly, these robust transcriptional oscillations are lost in the absence of the core clock gene *Bmal1* (Storch et al. 2007). In the liver, between 330 and 450 genes are expressed with a circadian rhythm (Panda et al. 2002a; Storch et al. 2002). In a creative use of conditional transgene expression, Ueli Schibler and colleagues knocked down the expression of the CLOCK/BMAL1 transcriptional oscillator exclusively in the liver. Remarkably, 31 genes in the clockless liver continued to oscillate presumably using systemic signals from the rest of the animal (Kornmann et al. 2007).

These systemic signals originating from the phase of the SCN that can drive and entrain rhythms of gene expression, and thus physiology, of peripheral oscillators are still being uncovered. They include signals from feeding, circulating humoral factors, and fluctuations in body temperature. The phase of the circadian rhythms of gene expression in the liver can be uncoupled from the rest of the body by providing food only when the animal would typically be asleep (Stokkan et al. 2001; Damiola et al. 2000). This food-induced resetting of peripheral oscillators is achieved, at least in part, by the ability of glucocorticoids in the circulatory system to control the phase of peripheral clocks (Damiola et al. 2000; Balsalobre et al. 2000). The Clara cells of the lung which are involved in detoxification of inhalants and production of various pulmonary secretions can also be entrained by glucocorticoids (Gibbs et al. 2009).

It is likely that just as various peripheral oscillators have fine-tuned their circadian transcriptomes, they also use unique combinations of physiologic phase cues for synchronization to the SCN's phase. The different rates of reentrainment among peripheral tissues to a new light–dark cycle suggest these distinctive properties (Yamazaki et al. 2000). However, there may be signals which are sufficient to control the phase of most tissues. For example, physiologic fluctuations in temperature can entrain all peripheral oscillators which have been examined (Brown et al. 2002; Buhr et al. 2010; Granados-Fuentes et al. 2004). The body temperature of mammals exhibits a circadian oscillation driven by the SCN regardless of sleep-activity state (Eastman et al. 1984; Scheer et al. 2005; Filipski et al. 2002; Ruby et al. 2002). Thus, light synchronizes the SCN to the external environment, and the SCN controls circadian fluctuations of body temperature. This SCN output serves as an input to the circadian clocks of peripheral tissues whose outputs are the various physiological and transcriptional rhythms seen within the local cells throughout the body. Fittingly, the SCN seems to be resistant to physiologic changes in body temperature (Brown et al. 2002; Buhr et al. 2010; Abraham et al. 2010). This would be an important feature of the system so that the phase of the SCN would not be influenced by the very parameter it was controlling. However, it is possible that the SCN may be sensitive to many cycles of cyclic temperature changes and that the SCN of some species may be more temperature sensitive than

others (Ruby et al. 1999; Herzog and Huckfeldt 2003). The intercellular coupling in the SCN responsible for these differences and possible mechanisms for temperature entrainment of peripheral tissues will be discussed in the following sections.

Further differences exist between the central pacemaker (SCN) and peripheral tissues at the level of the core molecular clock itself. The *Clock* gene was discovered as a hypomorphic mutation which caused the behavior of the animal and the molecular rhythms of the SCN to free-run at extremely long periods and become arrhythmic without daily entrainment cues (Vitaterna et al. 1994, 2006). However, if *Clock* is removed from the system as a null allele, the SCN itself and the behavior of the animal remain perfectly rhythmic (Debruyne et al. 2006). This is because the gene *Npas2* acts as a surrogate for the loss of *Clock* and compensates as the transcriptional partner of *Bmal1* (DeBruyne et al. 2007a). This compensatory role of *Npas2* only functions in the SCN, as the loss of *Clock* abolishes the circadian rhythmicity of the molecular oscillations in peripheral clocks (DeBruyne et al. 2007b). The SCN remains robustly rhythmic in the case of a loss of any single member of the negative limb of the transcriptional feedback cycle (Liu et al. 2007). The rhythms of peripheral clocks and dissociated cells remain rhythmic with the loss of *Cry2*; however, circadian rhythmicity is lost in peripheral tissues when *Cry1, Per1*, or *Per2* are removed (Liu et al. 2007). This importance of the *Per1* gene in these cellular rhythms is interesting in light of the subtle effect of the *Per1* null allele on behavior (Cermakian et al. 2001; Zheng et al. 1999). Adding further complexity, the combined removal of *Per1* and *Cry1* (two necessary negative limb components in peripheral tissues and single cells) reveals mice with normal free-running periods (Oster et al. 2003). Clearly differences exist between peripheral and the central oscillator both at the level of transcriptional circuitry and intercellular communication.

4 The SCN Is the Master Synchronizer in Mammals

The discovery of self-sustained circadian clocks in the cells of tissues throughout the body does not mean that the SCN should no longer be considered the “master” circadian clock. Although it does not drive the molecular rhythms in these cells, the SCN is necessary for the synchronization of phases among tissues to distinct phases (Yoo et al. 2004). The SCN does drive circadian rhythms of behavior such as activity–rest cycles and physiological parameters such as body temperature rhythms, as the 24-h component to these rhythms is lost when the SCN is lesioned (Stephan and Zucker 1972; Eastman et al. 1984). The behavioral rhythms of an SCN-lesioned animal can be restored by transplantation of donor SCN into the third ventricle (Drucker-Colín et al. 1984). The definitive proof that the SCN is the master clock for an animal’s behavior came when Michael Menaker and colleagues transplanted SCN from *tau*-mutant hamsters into SCN-lesioned wild-type hosts. The behavior of the host invariably ran with the free-running period of the donor SCN graft (Ralph et al. 1990).

The suprachiasmatic nuclei are paired structures of the ventral hypothalamus, with each half containing about 10,000 neurons in mice and about 50,000 neurons in humans (Cassone et al. 1988; Swaab et al. 1985). The most dorsal neurons of the SCN and their dorsal reaching efferents straddle the ventral floor of the third ventricle, and the most ventral neurons border the optic chiasm. Light information reaches the SCN from melanopsin-containing retinal ganglion cells (also called "intrinsically photosensitive retinal ganglion cells" or "ipRGCs") via the retinohypothalamic tract (RHT) (Moore and Lenn 1972; Berson et al. 2002; Hattar et al. 2002). The SCN receives retinal signals from rods, cones, and/or melanopsin; however, all light information which sets the SCN's phase is transmitted through the ipRGCs (Freedman et al. 1999; Panda et al. 2002b; Guler et al. 2008). Within the SCN, there are two main subdivisions known as the dorsomedial "shell" and the ventrolateral "core" (Morin 2007). These designations were originally defined due to distinct neuropeptide expression. The dorsomedial region is marked by high arginine–vasopressin (AVP) expression, and the ventrolateral region has high expression of vasoactive intestinal peptide (VIP) (Samson et al. 1979; Vandesande and Dierickx 1975; Dierickx and Vandesande 1977). This peptide expression is in addition to a mosaic of other peptides for which the expression and anatomical distinction varies among various species. For example, the mouse SCN also expresses gastrin-releasing peptide, enkephalin, neurotensin, angiotensin II, and calbindin, but the exact functions of each of these are unknown (Abrahamson and Moore 2001).

Another hallmark feature of the SCN is its circadian pattern of spontaneous action potentials [reviewed in Herzog (2007)]. The phase of neuronal firing is entrained by the light–dark cycle, but it also persists in constant darkness and as an ex vivo slice culture (Yamazaki et al. 1998; Groos and Hendriks 1982; Green and Gillette 1982). Similar to the induction of the *Per* genes by nocturnal light exposure, light pulses during the dark also cause an immediate induction of firing in the SCN (Nakamura et al. 2008). Just as the transcriptional clock can be observed in single cells, dissociated SCN neurons continue to fire action potentials with a circadian rhythm for weeks in vitro, although their phases scatter from one another (Welsh et al. 1995) (Fig. 2).

Synchrony of neurons within the SCN to each other is of paramount importance for the generation of a coherent output signal (see also Slat et al. 2013). At the onset of each circadian cycle, expression of the clock genes *Per1* and *Per2* starts in the most dorsomedial cells (AVP expressing) and the expression then spreads across each SCN towards the central and ventrolateral (VIP expressing) regions (Yan and Okamura 2002; Hamada et al. 2004; Asai et al. 2001). This medial-to-lateral, mirrored expression pattern is evident when gene expression in the SCN is viewed through in situ hybridization of fixed tissue or with visualization of gene reporters from a single organotypic culture (Asai et al. 2001; Yamaguchi et al. 2003). VIP signaling in particular seems key to maintaining synchrony among SCN neurons. Mice lacking VIP or its receptor $VPAC_2$ display erratic free-running behavior and the rhythms of individual neurons within a single SCN are no longer held in uniform phase (Harmar et al. 2002; Aton et al. 2005; Colwell et al. 2003). Rhythmic application of a $VPAC_2$ receptor

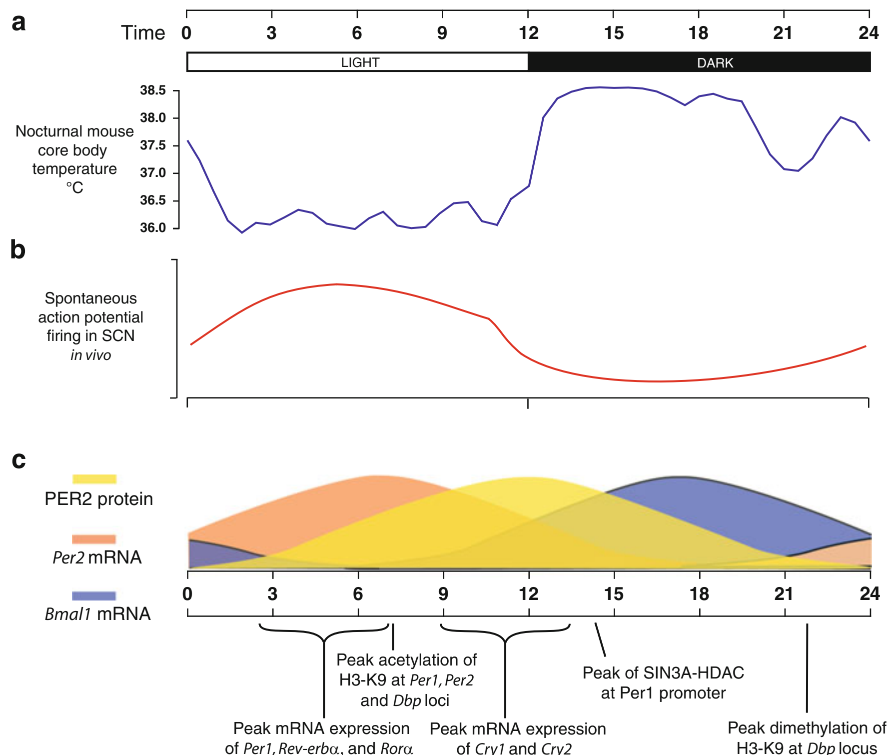

Fig. 2 Timing of circadian events in nocturnal rodents. (**a**) Mouse core body temperature as measured by radio telemetry. (**b**) Spontaneous firing rhythms from a cultured rat SCN as adapted from (Nakamura et al. 2008). (**c**) Molecular clock events are plotted schematically without axes for clarity. *Yellow sine wave* represents the phase of PER2 protein abundance in the mouse SCN. *Orange wave* represents the phase of *mPer2* mRNA abundance in the mouse SCN, and the *blue wave* represents the phase of *Bmal1* mRNA abundance. Chromatin information relates to the promoter regions of the *Per* genes and *Dbp* as reported by Etchegaray et al. (2003) and Ripperger and Schibler (2006). Sin3A-HDAC phase from Duong et al. (2011)

agonist to VIP$^{-/-}$ SCN neurons restores rhythmicity to arrhythmic cells and entrains the cells to a common phase (Aton et al. 2005). Application of purified VIP peptide into the SCN of animals in vivo causes phase shifts in free-running behavioral rhythms (Piggins et al. 1995). This VIP action on VPAC$_2$ receptors is mediated through cAMP signaling (An et al. 2011; Atkinson et al. 2011) which itself has been demonstrated as a determinant of phase and period in multiple tissues (O'Neill et al. 2008). The period of the whole SCN, and thus behavior, is determined by an averaging or an intermediate value of the periods of the individual neurons. In chimeric mice in which the SCN were comprised of various proportions of *Clock*$^{\Delta 19}$ (long free-running periods) and wild-type neurons, the free-running period of the mouse's behavior was determined by the proportion of wild-type to mutant cells (Low-Zeddies and Takahashi 2001).

Interestingly, the synaptic communication between cells in the SCN is necessary for the robust molecular oscillations of the core clock genes within individual cells. When intercellular communication via action potentials is lost by blocking voltage-gated Na^+ channels with tetrodotoxin (TTX), the circadian oscillations of *Per1* and *Per2* are greatly reduced and the synchrony of cells within the tissue loses phase coherence (Yamaguchi et al. 2003). When TTX is then removed, robust molecular oscillations resume and the cells resynchronize with the same intercellular phase profile as before the treatment (Yamaguchi et al. 2003). The amplitude of the molecular clock in an intact SCN allows the cells to overcome genetic and physiologic perturbations to which peripheral clocks are susceptible. For example, dissociated SCN neurons from $Cry1^{-/-}$ or $Per1^{-/-}$ mice lack circadian rhythm of clock gene expression; however, the intact SCN harboring these same mutations is as rhythmic as wild-type SCN with only period phenotypes (Liu et al. 2007). Even in the case of a severe clock gene mutation such as $Bmal1^{-/-}$ which causes a loss of circadian rhythmicity at the behavioral and single-cell level, the synaptic communication in an intact $Bmal1^{-/-}$ SCN allows for coordinated, but stochastic, expression of PER2 among SCN neurons (Ko et al. 2010).

The robustness of the intact SCN is also important for its ability to remain in appropriate phase in the presence of rhythmic physiologic perturbations. This is especially relevant in cases when an animal is exposed to situations that might uncouple aspects of behavior from a natural light–dark cycle. For example, when food availability is restricted to a time of the day when an animal is typically asleep and certain peripheral clocks shift their phase accordingly (as discussed in the previous section), the phase of the SCN remains tightly entrained to the light–dark cycle (Stokkan et al. 2001; Damiola et al. 2000). While body temperature fluctuations can entrain the rhythms of peripheral circadian clocks, the SCN can maintain its phase in the presence of physiologic temperature fluctuations (Brown et al. 2002; Buhr et al. 2010; Abraham et al. 2010). This is especially evident in cultured SCN where the tissue becomes sensitive to physiologic temperature changes when communication between cells is lost. Cells which hold their phase in the presence of temperature cycles as large as 2.5 °C in an intact SCN show exquisite sensitivity to temperature cycles as small as 1.5 °C when decoupled (Buhr et al. 2010; Abrahamson and Moore 2001). It should be noted that the above temperature data was collected in mice and in other species, such as rats, the temperature sensitivity of the SCN may be much greater (Ruby et al. 1999; Herzog and Huckfeldt 2003).

Most neurons in the SCN produce the neurotransmitter γ-aminobutyric acid (GABA) (Okamura et al. 1989). Daily administration of GABA to cultured dissociated SCN neurons can synchronize rhythms of spontaneous firing, and a single administration can shift their phase (Liu and Reppert 2000). GABA has also been implicated in conveying phase information between the dorsal and ventral portions exhibiting opposite acute effects on cells from these regions (Albus et al. 2005). However, other reports suggest that GABA signaling is not necessary for intra-SCN synchrony and even that GABA receptor antagonism increases firing rhythm amplitude (Aton et al. 2006). In fact, rhythmic application of a $VPAC_2$ agonist in a $Vip^{-/-}$ SCN was able to synchronize neuronal rhythms in the presence of chronic GABA-signaling blockade (Aton et al. 2006).

Along with internal synchrony, peptides and diffusible factors from the SCN are also important in the signaling from the SCN to the rest of the brain. The arrhythmic behavior of an SCN-lesioned animal could be rescued (at least partially) by the transplantation of a donor SCN encapsulated in a semipermeable membrane which allowed for passage of diffusible factors, but not neural outgrowth (Silver et al. 1996). The identity of this factor or factors is still being discovered. The SCN-secreted peptides transforming growth factor α (TGF-α), prokineticin 2 (PK2), and cardiotrophin-like cytokine (CLC) induce acute activity suppression and are rhythmically produced by the SCN (Kramer et al. 2001; Kraves and Weitz 2006; Cheng et al. 2002). Perhaps more behavioral activity inhibiting and maybe some activity-inducing factors will be identified in the future. It is likely that just as there is a mosaic of peptides produced locally in the SCN, the output signal involves a cocktail of secreted peptides along with direct neuronal efferents.

5 Temperature and Circadian Clocks

The influence of temperature on circadian clocks is important to discuss here both because of the ubiquity of temperature regulatory mechanisms in circadian clocks but also as potential targets for chronotherapeutics. First, as mentioned in the introduction to this chapter, all circadian rhythms are temperature compensated. This fundamental property allows the clock to maintain a stable period of oscillation regardless of the ambient temperature. A circadian clock would not be reliable if its period changed every time the sun went down or ran at a different period in the winter than in the summer. Temperature compensation is expressed as the coefficient Q_{10} which represents the ratio of the rate of a reaction at temperatures 10 °C apart. The Q_{10} of periods of various circadian rhythms of many species of broad phyla are between 0.8 and 1.2. Most chemical reactions within cells are affected by temperature; for example, most enzymatic reactions increase in rate as temperature is increased. In fact, the kinases CK1ε and δ increase their rate of phosphorylation of some protein targets at higher temperatures as would be expected; however, their rates of phosphorylation of clock proteins are stable at those same temperatures (Isojima et al. 2009). This temperature compensation is yet another example of the robustness of the molecular clock to retain precision in varying conditions. Even with broad reduction in global transcription, the clocks in mammalian cells remain rhythmic with only slightly shorter periods (Dibner et al. 2009).

The mechanisms of temperature compensation are still not understood, but great strides have been taken using the *Neurospora crassa* fungus. These organisms are routinely exposed to wide variations in temperature in their natural environment. The levels of the clock protein FRQ (which plays the negative limb role in fungus as PER and CRY do in mammals) are elevated at warmer temperatures and a long-form splice variant is observed at warm temperatures (Liu et al. 1997, 1998; Diernfellner et al. 2005). Mutants of the kinase CK-2, which phosphorylates FRQ, display either better temperature compensation than wild type or opposite

"overcompensation" (Mehra et al. 2009). In our own work, we observed an impairment in temperature compensation of PER2 rhythms in the SCN and pituitary of mice when the heat shock factors (HSF) were pharmacologically blocked (Buhr et al. 2010). These results fit with a model in which positive and negative effects of temperature on rates of cellular activity balance out to a net null effect. However, other findings suggest that this balancing model may be more complicated than necessary. Other extremely simple circadian rhythms, such as the in vitro phosphorylation of KaiC in *Synechococcus*, demonstrate beautiful temperature compensation with the presence of just the three proteins and ATP (Nakajima et al. 2005). Also, the transcription/translation-free rhythms of oxidation in peroxiredoxins in human red blood cells are temperature compensated (O'Neill and Reddy 2011). These results suggest that very simple oscillators may be temperature compensated purely by the robustness inherent in the individual processes rather than requiring balancing agents.

Although circadian clocks run at the same period at various temperatures, this does not mean that circadian clocks ignore temperature. Most species, particularly poikilothermic organisms, are exposed to wide daily temperature oscillations, and they use the change in temperature as an entraining cue. In fact, in *Neurospora* if a temperature cycle and light–dark cycle are out of phase, the fungus will entrain to the temperature cycle more strongly than to the light (Liu et al. 1998). In the fruit fly *Drosophila melanogaster*, the entrainment of global transcription rhythms appears to use a coordinated combination of light–dark cycles and temperature cycles so that the phase of light entrainment slightly leads the phase set by temperature of the same genes (Boothroyd et al. 2007). The importance of temperature changes is most strikingly observed at the behavioral level. In standard laboratory conditions with a light–dark cycle at a stable temperature, the flies show strong crepuscular activity with a large inactive period during the middle of the day. When more natural lighting is paired with a temperature cycle, the flies show a strong afternoon bout of activity and behaviorally act like a different species (Vanin et al. 2012).

Environmental temperature cycles act as extremely weak behavioral entrainment cues in warm-blooded animals, or "homeothermic" animals, which maintain their body temperature regardless of ambient temperature (Rensing and Ruoff 2002). However, the internal body temperature of homeothermic animals undergoes circadian fluctuations with amplitudes of approximately 1 °C and 5 °C depending on the species (Refinetti and Menaker 1992). As mentioned earlier, the surgical ablation of the SCN abolishes the circadian component to body temperature fluctuation along with behavioral and sleep rhythms in mice, rats, and ground squirrels (Eastman et al. 1984; Filipski et al. 2002; Ruby et al. 2002). Although it is hard to isolate effects that activity, sleep, and the SCN have on body temperature oscillations, both human and rodent examples exist. In humans, the circadian oscillation of rectal temperature persists if a person is restricted to 24-h bed rest and is deprived of sleep (Aschoff 1983). In hibernatory animals, such as the ground squirrel, a low-amplitude SCN-driven body temperature rhythm is observed during bouts of hibernation in which there is an absence of activity for days at a time (Ruby et al. 2002; Grahn et al. 1994).

As discussed in the Peripheral Clocks section, these rhythms of body temperature fluctuation are sufficient to entrain the peripheral oscillators of homeothermic animals in all cases that have been reported (Brown et al. 2002; Buhr et al. 2010; Granados-Fuentes et al. 2004; Barrett and Takahashi 1995). The most recent evidence suggests that this effect on the molecular clock mammals by temperature cycles is regulated by the heat shock pathway. Briefly, after heat exposure, the heat shock factors (HSF1, HSF2, and HSF4) initiate the transcription of genes with heat shock elements (HSE) in their promoters (Morimoto 1998). The genes of heat shock proteins (HSP) contain HSEs, and once translated, these proteins chaperone or sequester the HSFs from further transcription. This feedback loop maintains a transient response to temperature changes. Although commonly associated with heat tolerance to extreme temperatures, the dynamic range heat shock pathway can include temperature changes within the physiologic range (Sarge et al. 1993). Blocking HSF transcription transiently with the pharmacological agent KNK437 mimicked the phase shifts caused by a cool temperature pulse and blocked the phase-shifting effects of warm pulses (Buhr et al. 2010). Also, a brief exposure to warm temperatures caused an acute reduction of *Per2* levels followed by an induction when returned to a cooler temperature in the liver (Kornmann et al. 2007). Along with being a temperature sensor for phase setting, it is also evident that the HSF family and the circadian clock are more intimately related. Although the levels of HSF proteins have not been found to have a circadian oscillation, their binding to target motifs certainly does even in the absence of temperature cycles (Reinke et al. 2008). Additionally, the promoter of the *Per2* gene contains HSEs that are conserved among multiple species, and a number of *hsp* genes oscillate with a phase similar to *Per2* (Kornmann et al. 2007). Finally, deletion of the *Hsf1* gene lengthens the free-running behavioral period of mice by about 30 min, and pharmacologic blockade of HSF-mediated transcription ex vivo causes the molecular clock to run >30 h in SCN and peripheral tissues (Buhr et al. 2010; Reinke et al. 2008). Clearly the heat shock response pathway exerts both phase and period influence on the circadian clock. It will be exciting to see how this relationship is further elucidated in the future.

6 Conclusions and Summary

The circadian system of all organisms contain a core oscillator, a way by which this clock can be set by the environment, and output behaviors or processes whose phases are determined by the core clock. This can be observed as an animal in its environment synchronizes its behavior to the sun or as a cell in the liver synchronizes its metabolic state to the phase of the SCN. The precision of the system allows for perfectly timed oscillations throughout the body of a well-functioning organism or sets the stage for mistimed events and disease in a malfunctioning system. Much has been learned about the molecular function of the clock itself and the ways by which clocks within a single organism

communicate, but more insights are uncovered monthly. The field is now at the level where serious therapeutic strategies can be developed and implemented for the treatment of sleep and metabolic disorders, optimizing timing of drug delivery, and the co-option of circadian elements to control various cellular pathways and vice versa.

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The Epigenetic Language of Circadian Clocks

Saurabh Sahar and Paolo Sassone-Corsi

Abstract Epigenetic control, which includes DNA methylation and histone modifications, leads to chromatin remodeling and regulated gene expression. Remodeling of chromatin constitutes a critical interface of transducing signals, such as light or nutrient availability, and how these are interpreted by the cell to generate permissive or silenced states for transcription. CLOCK-BMAL1-mediated activation of clock-controlled genes (CCGs) is coupled to circadian changes in histone modification at their promoters. Several chromatin modifiers, such as the deacetylases SIRT1 and HDAC3 or methyltransferase MLL1, have been shown to be recruited to the promoters of the CCGs in a circadian manner. Interestingly, the central element of the core clock machinery, the transcription factor CLOCK, also possesses histone acetyltransferase activity. Rhythmic expression of the CCGs is abolished in the absence of these chromatin modifiers. Here we will discuss the evidence demonstrating that chromatin remodeling is at the crossroads of circadian rhythms and regulation of metabolism and cellular proliferation.

Keywords Circadian clock • Epigenetics • Histone modifications • Sirtuins

1 Introduction

Circadian rhythms occur with a periodicity of about 24 h and regulate a wide array of metabolic and physiologic functions. Accumulating epidemiological and genetic evidence indicates that disruption of circadian rhythms can be directly linked to many pathological conditions, including sleep disorders, depression, metabolic

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syndrome, and cancer. Intriguingly, a number of molecular gears constituting the clock machinery have been found to establish functional interplays with regulators of cellular metabolism and cell cycle.

The Earth's rotation around its axis leads to day–night cycles, which affects the physiology of most living organisms. Circadian (from the Latin *circa diem* meaning "about a day") clocks are intrinsic, time-tracking systems that enable organisms to anticipate environmental changes (such as food availability and predatory pressure) and allow them to adapt their behavior and physiology to the appropriate time of day (Schibler and Sassone-Corsi 2002). Feeding behavior, sleep–wake cycles, hormonal levels, and body temperature are just a few examples of physiological circadian rhythms, with light being the principal zeitgeber ("time giver"). Other zeitgebers, such as feeding time and temperature, are discussed in accompanying chapters in this book (Brown and Azzi 2013; Buhr and Takahashi 2013).

The three integral parts of circadian clocks are the following: an input pathway that includes detectors to receive environmental cues (or zeitgebers) and transmits them to the central oscillator; a central oscillator that keeps circadian time and generates rhythm; and output pathways through which the rhythms are manifested via control of various metabolic, physiological, and behavioral processes. Distinguishing characteristics of circadian clocks include that they are entrainable (synchronizable by external cues), self-sustained (oscillations can persist even in the absence of zeitgebers), and temperature compensated (moderate variations in ambient temperature does not affect the period of circadian oscillation) (Merrow et al. 2005).

Circadian clocks are present in almost all of the tissues in mammals. The master or "central" clock is located in the hypothalamic suprachiasmatic nucleus (SCN), which contains 10–15,000 neurons (Slat et al. 2013). Peripheral clocks are present in almost all other mammalian tissues such as liver, heart, lung, and kidney, where they maintain circadian rhythms and regulate tissue-specific gene expression (Brown and Azzi 2013). These peripheral clocks are synchronized by the central clock to ensure temporally coordinated physiology. The synchronization mechanisms implicate various humoral signals, including circulating entraining factors such as glucocorticoids. The SCN clock can function autonomously, without any external input, but can be set by environmental cues such as light. The molecular machinery that regulates these circadian rhythms comprises of a set of genes, known as "clock" genes, whose products interact to generate and maintain rhythms (Buhr and Takahashi 2013).

A conserved feature among many organisms is the regulation of the circadian clock by a negative feedback loop (Sahar and Sassone-Corsi 2009). Positive regulators induce the transcription of clock-controlled genes (CCGs), some of which encode proteins that feedback on their own expression by repressing the activity of positive regulators. CLOCK and BMAL1 are the positive regulators of the mammalian clock machinery which regulate the expression of the negative regulators: cryptochrome (*CRY1* and *CRY2*) and period (*PER1*, *PER2*, *PER3*) families. CLOCK and BMAL1 are transcription factors that heterodimerize through the PAS domain and induce the expression of clock-controlled genes by binding to their promoters at E-boxes [CACGTG]. Once a critical concentration of the PER

and CRY proteins is accumulated, these proteins translocate into the nucleus and form a complex to inhibit CLOCK-BMAL1-mediated transcription, thereby closing the negative feedback loop. In order to start a new transcriptional cycle, the CLOCK-BMAL1 complex needs to be derepressed through the proteolytic degradation of PER and CRY. Core clock genes (such as *Clock*, *Bmal1*, *Period*, *Cryptochrome*) are necessary for generation of circadian rhythms, whereas CCGs (such as *Nampt*, *Alas1*) are regulated by the core clock genes.

Some CCGs are transcription factors, such as albumin D-box-binding protein (DBP), RORα, and REV-ERBα, which can then regulate cyclic expression of other genes. DBP binds to D-boxes [TTA(T/C)GTAA], whereas RORα and REV-ERBα bind to the *Rev-Erb/ROR*-binding element, or RRE [(A/T)A(A/T)NT(A/G) GGTCA]. Approximately 10 % of the transcriptome displays robust circadian rhythmicity (Akhtar et al. 2002; Panda et al. 2002). Interestingly, most transcripts that oscillate in one tissue do not oscillate in another (Akhtar et al. 2002; Miller et al. 2007; Panda et al. 2002).

2 Epigenetics and the Circadian Clock

"Epigenetics" literally means "above genetics." It is defined as the study of heritable changes in gene expression that does not involve any change to the DNA sequence. Such changes in gene expression can be brought about by a variety of mechanism that involves a combination of posttranslational modifications of histones, remodeling of chromatin, incorporation of histone variants, or methylation of DNA on CpG islands. Histone acetylation is a mark for activation of transcription, which is achieved by remodeling the chromatin to make it more accessible to the transcription machinery (Jenuwein and Allis 2001). Histone methylation, on the other hand, acts as a signal for recruitment of chromatin remodeling factors which can either activate or repress transcription. DNA methylation leads to compaction of the chromatin and causes gene silencing. Many of these epigenetic events are crucial in regulation of cellular metabolism and survival.

Genes encoding circadian clock proteins are regulated by epigenetic mechanisms, such as histone phosphorylation, acetylation, and methylation, which have been shown to follow circadian rhythm (Crosio et al. 2000; Etchegaray et al. 2003; Masri and Sassone-Corsi 2010; Ripperger and Schibler 2006). The first study demonstrating that chromatin remodeling is involved in circadian gene expression reported that exposure to light causes rapid phosphorylation of histone 3 on serine 10 (H3-S10) in the SCN (Crosio et al. 2000). This phosphorylation parallels induction of immediate early genes such as *c-fos* and *Per1*, thereby indicating that light-mediated signaling can regulate circadian gene expression by remodeling the chromatin (Crosio et al. 2000).

CLOCK-BMAL1-mediated activation of CCGs has been shown to be coupled to circadian changes in histone acetylation at their promoters (Etchegaray et al. 2003). The central element of the core clock machinery, the transcription factor CLOCK,

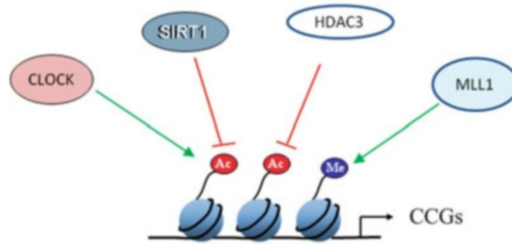

Fig. 1 Epigenetic regulation of gene expression by circadian clock CLOCK can acetylate histones to induce gene expression. CLOCK interacts with MLL1 (a histone methyltransferase) and SIRT1 (a deacetylase). These epigenetic regulators can modify the chromatin according the environmental stimuli, such as nutrient availability. Furthermore, REV-ERBα, a clock-controlled gene, can cause recruitment of HDAC3 and deacetylate histones. Circadian regulation of either the expression or the activity of these epigenetic regulators determines whether the gene gets turned "ON" (*green arrows*) or "OFF" (*red arrows*)

also possesses intrinsic histone acetyltransferase (HAT) activity (Doi et al. 2006). Since CLOCK binds to E-box regions of DNA, the HAT activity of CLOCK can selectively remodel chromatin at the promoters of CCGs and is essential for circadian gene expression (Fig. 1). The enzymatic activity of CLOCK also allows it to acetylate nonhistone substrates such as its own binding partner, BMAL1 (Hirayama et al. 2007). CLOCK specifically acetylates BMAL1 at a conserved residue, an event that facilitates CRY-dependent repression.

Histone methylation is also important for circadian gene expression. Mixed lineage leukemia 1 (MLL1), a methyltransferase that methylates histone H3 at lysine 4 (H3K4), associates with CLOCK and is recruited to promoters of CCGs in a circadian manner (Fig. 1) (Katada and Sassone-Corsi 2010). H3K4 methylation at these promoters also displays rhythmicity (Katada and Sassone-Corsi 2010). H3K4 methylation has been intimately linked to transcriptional activation. Lysine residues can be mono-, di-, or trimethylated at the ε-amino group, with each state correlating with a distinct functional effect. Dimethylated H3K4 (H3K4me2) occurs at both inactive and active euchromatic genes, whereas H3K4me3 is present prominently at actively transcribed genes and is widely accepted as a unique epigenetic mark that defines an active chromatin state in most eukaryotes. It is thereby noteworthy that MLL1 is specifically involved in trimethylation (Katada and Sassone-Corsi 2010). Notably, H3K4 methylation has often been shown to be associated with specific H3 Lys9 (H3K9) and Lys14 (H3K14) and H4 Lys16 (H4K16) acetylation, and these are all "marks" associated with active gene expression (Ruthenburg et al. 2007).

2.1 Role of SIRT1 in Regulation of Circadian Rhythms

The finding of a circadian HAT opened the search for a counterbalancing histone deacetylase (HDAC). Recently, SIRT1 was identified to be a modulator of the circadian clock machinery (Asher et al. 2008; Nakahata et al. 2008). SIRT1 belongs

to the family of sirtuins, which constitutes the so-called class III of HDACs. These are HDACs whose enzymatic activity is NAD^+ dependent and that has been directly linked to the control of metabolism and aging (Bishop and Guarente 2007). SIRT1 plays crucial roles in metabolism by (a) deacetylating several proteins that participate in metabolic pathways and (b) regulating gene expression by histone deacetylation. Since the NAD^+/NADH ratio is a direct measure of the energy status of a cell, the NAD^+ dependence of SIRT1 directly links cellular energy metabolism and deacetylation of target proteins (Imai et al. 2000). Recently, two independent studies identified SIRT1 to be a critical modulator of the circadian clock machinery (Asher et al. 2008; Nakahata et al. 2008). While Asher et al. observed oscillations in SIRT1 protein levels (Asher et al. 2008), Nakahata et al. demonstrated that SIRT1 activity, and not its protein levels, oscillates in a circadian manner (Nakahata et al. 2008). Circadian oscillations in NAD^+ levels were later shown to drive SIRT1 rhythmic activity (Nakahata et al. 2009). SIRT1 modulates circadian rhythms by deacetylating histones (histone H3 Lys9 and Lys14 at promoters of rhythmic genes) and nonhistone proteins (BMAL1 and PER2). The CLOCK-BMAL1 complex interacts with SIRT1 and recruits it to the promoters of rhythmic genes (Fig. 1). Importantly, circadian gene expression and BMAL1 acetylation are compromised in liver-specific SIRT1 mutant mice (Nakahata et al. 2008). While BMAL1 acetylation acts as a signal for CRY recruitment (Hirayama et al. 2007), PER2 acetylation enhances its stability (Asher et al. 2008). These findings led to the concept that SIRT1 operates as a rheostat of the circadian machinery, modulating the amplitude and "tightness" of CLOCK-mediated acetylation and consequent transcription cycles in metabolic tissues (Nakahata et al. 2008).

Circadian oscillation of SIRT1 activity suggested that cellular NAD^+ levels may also oscillate. Circadian clock controls the expression of nicotinamide phosphoribosyltransferase (NAMPT), a key rate-limiting enzyme in the salvage pathway of NAD^+ biosynthesis (Nakahata et al. 2009; Ramsey et al. 2009). The rhythmicity in the expression of this enzyme drives the oscillation in NAD^+ levels (Nakahata et al. 2009; Ramsey et al. 2009). CLOCK, BMAL1, and SIRT1 are recruited to the *Nampt* promoter in a circadian time-dependent manner (Fig. 2). The oscillatory expression of *Nampt* is abolished in *Clock*/*Clock* mice, which results in drastically reduced levels of NAD^+ in MEFs derived from these mice (Nakahata et al. 2009). These results make a compelling case for the existence of an enzymatic/transcriptional feedback loop, wherein SIRT1 regulates the levels of its own cofactor. Interestingly, mice deficient of NAD^+ hydrolase CD38 displayed altered rhythmicity of NAD^+. Very high levels of NAD^+ in tissues such as the brain and liver have been reported in the CD38-null mice (Aksoy et al. 2006). The high, chronic levels of NAD^+ result in several anomalies in circadian behavior and metabolism (Sahar et al. 2011). CD38-null mice display a shortened period length of locomotor activity and alteration in the rest–activity rhythm (Sahar et al. 2011).

SIRT1 also deacetylates and thereby regulates several proteins involved in the regulation of metabolism and cell proliferation (Fig. 2). For example, SIRT1 regulates gluconeogenesis by deacetylating and activating PPARγ-coactivator α (PGC1α) and Forkhead box O1 (FOXO1) (Schwer and Verdin 2008). FOXO1