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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 IV Systems Biology of Circadian Clocks

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

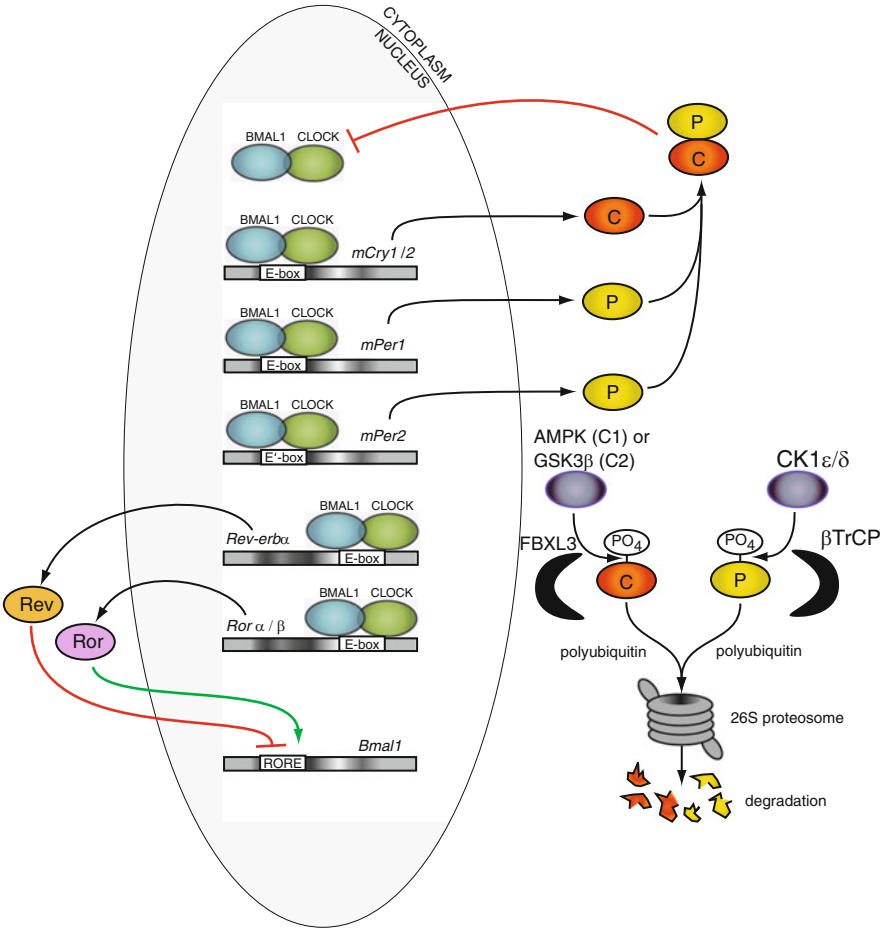

Fig. 1 Schematic of the molecular clock of mammals. CLOCK/BMAL1 heterodimers (*green* and *blue ovals*) bind DNA of clock target genes at E-boxes or E′-boxes and initiate the transcription of their RNA. The resulting PER and CRY proteins (*red* and *yellow ovals*) dimerize in the cytoplasm and translocate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

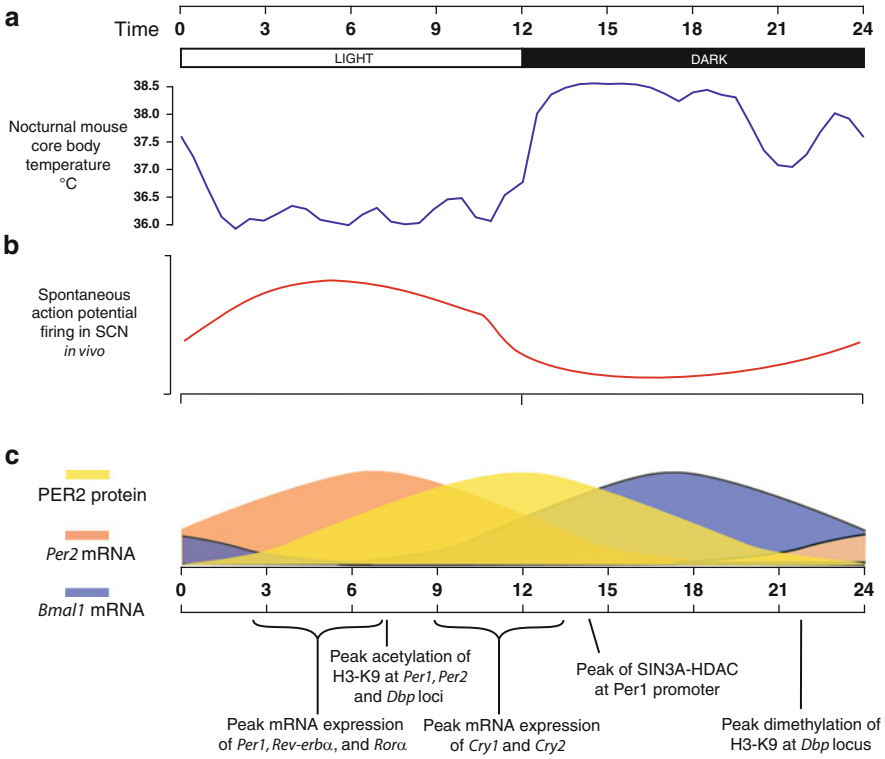

Fig. 2 Timing of circadian events in nocturnal rodents. (**a**) Mouse core body temperature as measured by radio telemetry. (**b**) Spontaneous firing rhythms from a cultured rat SCN as adapted from (Nakamura et al. 2008). (**c**) Molecular clock events are plotted schematically without axes for clarity. *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,

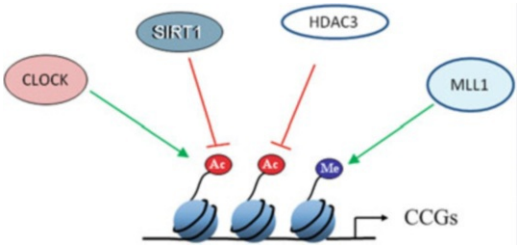

Fig. 1 Epigenetic regulation of gene expression by circadian clock CLOCK can acetylate histones to induce gene expression. CLOCK interacts with MLL1 (a histone methyltransferase) and SIRT1 (a deacetylase). These epigenetic regulators can modify the chromatin according the environmental stimuli, such 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

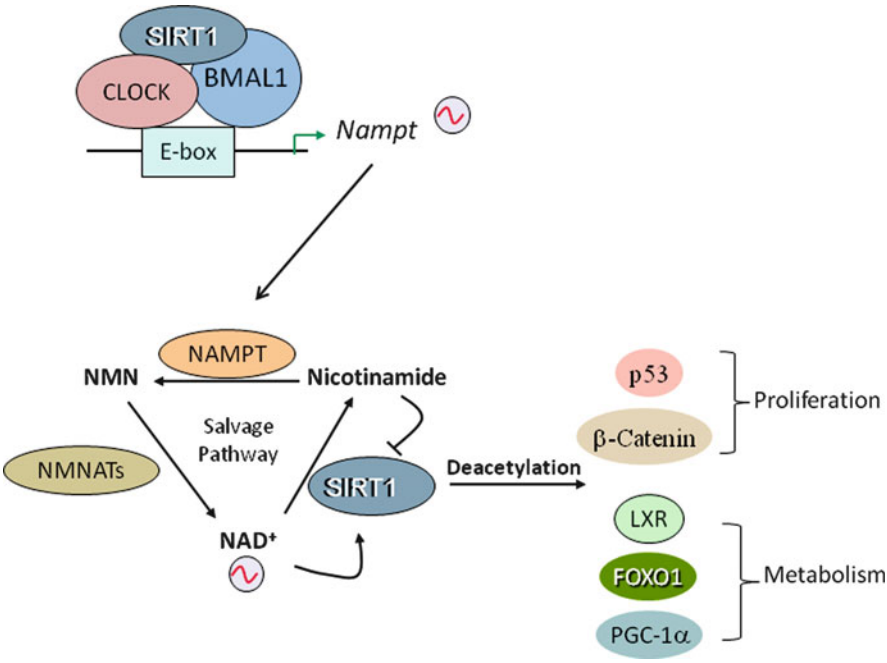

Fig. 2 SIRT1: a circadian regulator The circadian clock controls the expression of nicotinamide phosphoribosyltransferase (*Nampt*), the rate-limiting enzyme in mammalian NAD^+ biosynthesis from nicotinamide. NAMPT catalyzes the transfer of a phosphoribosyl residue from 5-phosphoribosyl-1-pyrophosphate (PRPP) to nicotinamide to produce nicotinamide mononucleotide (NMN), which is then converted to NAD^+ by nicotinamide mononucleotide adenylyltransferases (there are three Nmnat genes). Oscillation in NAMPT results in circadian variations in NAD^+ levels, which determines the activity of SIRT1. Thus, SIRT1 determines the oscillatory levels of its own coenzyme, NAD^+. SIRT1 can also deacetylate and regulate proteins involved in metabolism and cell proliferation

directly regulates expression of several gluconeogenic genes (Frescas et al. 2005), whereas PGC1α coactivates glucocorticoid receptors and hepatic nuclear factor 4-alpha (HNF-4α) to induce the expression of gluconeogenic genes (Yoon et al. 2001). SIRT1 also regulates cholesterol metabolism by deacetylating, and thus activating, Liver X receptor (LXR) (Li et al. 2007) (Fig. 2). LXR regulates cholesterol metabolism by inducing the expression of the ATP-binding cassette transporter A1 (*Abca1*), which mediates cholesterol efflux from peripheral tissues to the blood. Furthermore, it seems evident that SIRT1 may promote or prevent cancer depending on the specific function of its substrate. By deacetylating and thereby inactivating β-catenin, SIRT1 may lead to reduced cell proliferation (Firestein et al. 2008). SIRT1 deacetylates p53 and thus inhibits its activity (Vaziri et al. 2001), resulting in reduced apoptosis after genotoxic stress (Fig. 2). Since SIRT1 activity is regulated in a circadian manner, it would be interesting to determine if the acetylation of other SIRT1 targets oscillates in a circadian manner.

2.2 The Complexity of the Circadian Epigenome

Accumulating evidence shows that a variety of chromatin remodelers contribute to various aspects of the circadian epigenome (Masri and Sassone-Corsi 2010). In addition, the circadian machinery appears to occupy a pivotal position in linking metabolism to epigenetics (Katada et al. 2012). Histone deacetylase 3 (HDAC3) is a deacetylase that has recently been shown to modulate histone acetylation of circadian genes, particularly those that are responsible for lipid metabolism. The regulatory function of REV-ERBα is controlled by the nuclear receptor corepressor 1 (NCoR1), a corepressor that recruits HDAC3 to mediate transcriptional repression of target genes, such as *Bmal1*. When the NCoR1-HDAC3 association is genetically disrupted in mice, circadian and metabolic defects develop (Alenghat et al. 2008). These mice demonstrate a shorter period, increased energy expenditure and are resistant to diet-induced obesity (Alenghat et al. 2008). HDAC3 recruitment to the genome was recently shown to be rhythmic in liver (high during the day and low at night) (Feng et al. 2011). At these HDAC3 binding sites, REV-ERBα and NCoR1 recruitment were in phase with HDAC3 recruitment, whereas histone acetylation and RNA polymerase II recruitment were anti-phasic. Depletion of either HDAC3 or REV-ERBα was shown to cause fatty liver phenotype, such as increased hepatic lipid and triglyceride content (Feng et al. 2011).

HDAC1 was also shown to form a complex with SIN3A (a protein that modulates transcription by interacting with transcription repressors) and PER2, and it is known to be recruited to *Per1* promoter. HDAC1 can then deacetylate the histones and repress transcription of *Per1*. Depletion of SIN3A from synchronized fibroblasts caused a shortening of circadian period length (Duong et al. 2011).

Although the identity of a circadian histone demethylase is currently unknown, JARID1a, a histone demethylase, has been recently shown to regulate circadian gene expression (DiTacchio et al. 2011). Surprisingly, the histone demethylase activity of this enzyme is not required for its regulation of circadian rhythms.

Altogether, these findings underscore the importance of epigenetic mechanisms in circadian regulation and reveal the molecular pathways by which such essential control is achieved.

3 Circadian Disruption and Disease: Cancer and Metabolic Disorders

Circadian control of physiology and behavior is required for a healthy life. Disruption of circadian rhythms has been considered as a causative factor for development of several diseases. As discussed below, mutation in circadian clock proteins that either have histone-modifying ability (such as CLOCK) or associate with histone modifiers (such as BMAL1, PER2, and REV-ERBα) has been linked to cancer and metabolic syndrome (Sahar and Sassone-Corsi 2012).

3.1 Mutations in the Clock Machinery and Cancer Association

A number of epidemiological studies have linked defects in circadian rhythms to increased susceptibility to develop cancer and poor prognosis. This evidence is supported by gene expression studies. For example, the expression of all three *Per* genes is deregulated in breast cancer cells (Chen et al. 2005). *PER1* expression is downregulated in most patients, possibly due to methylation of its promoter. Mutations in *NPAS2* have been associated with increased risk for breast cancer and non-Hodgkin's lymphoma (Hoffman et al. 2008). More importantly, a number of studies using mouse models have established convincing links between some clock genes and tumorigenesis. Specifically, *Per1* and *Per2* appear to act as tumor suppressors in mice (Fu et al. 2002; Gery et al. 2006). Targeted ablation of *Per2* leads to the development of malignant lymphomas (Fu et al. 2002), whereas its ectopic expression in cancer cell lines results in growth inhibition, cell cycle arrest, apoptosis, and loss of clonogenic ability (Gery et al. 2005). Interestingly, *Per2* mRNA levels are downregulated in several human lymphoma cell lines and acute myeloid leukemia patients (Gery et al. 2005). Overexpression of *Per1* can also suppress growth of human cancer cell lines (Gery et al. 2006). Furthermore, *PER1* mRNA levels are also downregulated in non-small cell lung cancer tissues compared to matched normal tissues (Gery et al. 2006). In addition, knockdown of *CK1ε* induces growth inhibition of cancer cells, and *CK1ε* expression is increased in various human cancers, such as leukemia and prostate cancer (Yang and Stockwell 2008). These results consistently point toward a direct link between the dysfunction of key circadian regulators and cancer (Sahar and Sassone-Corsi 2007).

An interesting link between circadian clock and breast cancer was established in a study demonstrating that PER2 can bind to and destabilize estrogen receptor α (ERα) (Gery et al. 2007), a key transcription factor that promotes growth of mammary epithelial cells and whose dysregulated activity is known to cause breast cancer (Green and Carroll 2007). Consequently, *Per2* overexpression leads to reduced ERα protein levels and transcriptional activity.

It is important to note that mutation of one or more core clock genes is itself not necessarily sufficient to elicit enhanced tumor incidence. In addition, there is no apparent correlation between the disruption of circadian behavior and increased tumorigenesis in mouse models of circadian rhythms. Indeed, $Cry1^{-/-}Cry2^{-/-}$ mice (Gauger and Sancar 2005) or *Clock*/*Clock* mutant mice (Antoch et al. 2008), whose circadian rhythms are highly compromised, do not show a predisposition to cancer upon irradiation. Moreover, MEFs derived from *Clock*/*Clock* mutant mice display reduced DNA synthesis and cell proliferation compared to wild-type MEFs (Miller et al. 2007). Somewhat unexpectedly, ablation in the mouse of both *Cry* genes in a $p53^{-/-}$ background delays the onset of cancer (Ozturk et al. 2009). These notions may suggest that other regulatory features intrinsic to clock regulators, independent of their circadian function, could participate in carcinogenesis. It seems that individual core circadian clock proteins (such as PER1, PER2) might have acquired multiple roles and hence can control both rhythms and cell cycle.

Also, the consequence of circadian disruption on cancer predisposition might be dependent on how the rhythm is disrupted.

The molecular mechanism of how circadian clock influences cancer development and progression could be explained by its regulation of cell cycle, DNA damage response, and cellular metabolism (Hunt and Sassone-Corsi 2007; Antoch and Kondratov 2013). Circadian regulation of genes encoding key cell cycle regulators, such as *Wee1* (G_2/M transition) (Matsuo et al. 2003), c-*myc* (G_0/G_1 transition) (Fu et al. 2002), and *Cyclin D1* (G_1/S transition) (Fu et al. 2002), has been demonstrated in mammals, and light induces the expression of *Wee1* in zebra fish (Hirayama et al. 2005). WEE1 is a kinase that phosphorylates and inactivates the CDC2/cyclin B1 complex to control G_2/M transition during mitosis. *Wee1* displays robust CLOCK-BMAL1 dependent circadian oscillations in the mouse liver (Matsuo et al. 2003). Furthermore, partial hepatectomy-induced liver regeneration is impaired in *Cry*-deficient arrhythmic mice, which also show deregulated expression of *Wee1* (Matsuo et al. 2003). These studies indicate that WEE1 may function as a key molecular link between circadian and cell cycles.

Damage to cellular DNA, either by intracellular agents (such as metabolic byproducts) or external agents (such as ionizing radiations), can cause cancer. However, cells have evolved several mechanisms to repair the damaged DNA. Recent results suggest that one such repair mechanism, the nucleotide excision repair pathway, displays circadian oscillation in mouse brain, possibly through oscillation in the expression of the DNA damage-recognition protein xeroderma pigmentosum A (XPA) (Kang et al. 2009). XPA levels also oscillate in mouse liver (Kang et al. 2009), suggesting that the circadian nucleotide excision repair might also be operating in peripheral tissues. Confirming this notion, a recent study found that XPA protein levels and the rate of excision repair oscillate in a circadian manner in mouse skin (Gaddameedhi et al. 2011). Consequently, mice are more susceptible to skin cancer when exposed to ultraviolet radiation in the morning when the rate of DNA repair is lower (Gaddameedhi et al. 2011).

Finally, circadian clock proteins, such as PER1 and Timeless (TIM), interact with key checkpoint proteins (Gery et al. 2006; Unsal-Kacmaz et al. 2005). It is conceivable that uncoupling of this delicate balance could induce DNA damage, predisposing cells to tumorigenesis.

3.2 Cancer Chronotherapy

Chronotherapy refers to the administration of drugs at a certain time of the day when its efficacy is the highest and the side effects are the lowest (see also Ortiz-Tudela et al. 2013). An example of successful chronotherapy is the use of the cholesterol-lowering drugs statins. Statins inhibit HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. The expression of HMG-CoA reductase displays circadian rhythmicity, being highest at night. Hence, statins are most effective when administered before bedtime. Chronotherapy has also shown promise

in treating cancer. It is widely accepted that cells enter various phases of cell cycle in a circadian manner. Fast-growing or advanced tumors become asynchronous with the host cells and display ultradian (less than 24 h) rhythms (Lis et al. 2003). In an elegant experiment, Klevecz et al. demonstrated that the proliferation of tumor and non-tumor cells from ovarian cancer patients significantly differed in their peak S phase (Klevecz et al. 1987). Similar observations in other types of cancer [such as non-Hodgkin's lymphoma (Smaaland et al. 1993)] suggest that there is a possible window of time when a cytotoxic drug would kill the tumor cells more effectively than the noncancerous host cells. More than 30 anticancer drugs have been found to vary in toxicity and efficacy by more than 50 % as a function of time of administration in various experimental models (Levi et al. 2007). In clinical studies several anticancer drugs, such as 5-fluorouracil (5-FU) and platinum complex analogs that are specifically toxic to replicating cells, have been shown to be more efficacious and less toxic when administered at a specific circadian time (Levi et al. 2007). For example, chronotherapy using doxorubicin and cisplatin showed significant improvement in survival rate of patients with ovarian cancer when doxorubicin was administered in the morning followed by cisplatin 12 h later (Kobayashi et al. 2002). Further studies are needed to identify the molecular mechanisms responsible for the beneficial effects of circadian administration of anticancer drugs. Another report demonstrated that sensitivity to cyclophosphamide, an anticancer drug, varies greatly in wild-type mice depending upon the time of administration (Gorbacheva et al. 2005). However, *Clock*/*Clock* mutant mice and *Bmal1*$^{-/-}$ mice are more sensitive and did not display variation in sensitivity at different times, indicating dependency on clock components, whereas *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice are more resistant to cyclophosphamide. These results suggest that activities of the core clock components have direct manifestation in response to genotoxic stress induced by anticancer drugs.

3.3 Circadian Disruption and Metabolic Disorders

Shift work, and accompanying light exposure at night, has been implicated in the development of metabolic syndrome and cardiovascular diseases (De Bacquer et al. 2009; Karlsson et al. 2001; Marcheva et al. 2013). A recent study showed that mice exposed to light at night gained more weight, had reduced glucose tolerance, and ate more during the light phase. Interestingly, when food was restricted to the dark phase, weight gain was prevented (Fonken et al. 2010). In another study, mice fed a high-fat diet only during the light phase gained more weight when compared to mice that ate the same high-fat diet but only during the dark phase, an observation that highlights the importance in the timing of food intake (Arble et al. 2009). These results raise an interesting question: Could adjusting our food intake exclusively during the active phase (daytime for humans) be an effective way of weight control? Since humans have evolved for thousands of years without artificial light, our internal clock still functions the best when natural light is the only source

of light, so it is conceivable that restricting food intake to daytime may help control weight gain.

Not just the timing but the quality of the diet might also affect the clock. Mice fed a high-fat diet had altered circadian rhythms and displayed a lengthening of the period of locomotor activity (Kohsaka et al. 2007). Interestingly, these mice also consumed a higher-than-normal percentage of food during the light phase. Moreover, the expression of core clock genes and the clock-controlled genes (CCGs) was altered in the mice that were fed a high-fat diet (Kohsaka et al. 2007). These studies have clearly established that metabolism can also control peripheral clocks.

If the circadian machinery is critical for metabolic homeostasis, deletion or mutation of individual core clock components or of CCGs should lead to metabolic disorders. This is indeed the case as illustrated by examples discussed below.

3.3.1 CLOCK and BMAL1

Loss of function of CLOCK and BMAL1, the central transcription factors that regulate circadian rhythms, leads to several metabolic anomalies. *Clock/Clock* mutant mice, which are arrhythmic when placed in constant darkness, become hyperphagic and obese and develop classical signs of "metabolic syndrome" such as hyperglycemia, dyslipidemia, and hepatic steatosis (fatty liver) (Turek et al. 2005). In addition, the mRNA levels of the neuropeptides orexin and ghrelin—both involved in the neuroendocrine regulation of food intake (Adamantidis and de Lecea 2009; Saper et al. 2002)—are also reduced in these mice. Furthermore, renal sodium reabsorption is compromised and arterial blood pressure is reduced in the $Clock^{-/-}$ mice (Zuber et al. 2009). Loss of BMAL1, which renders mice completely arrhythmic (Bunger et al. 2000), also leads to disruption of oscillations in glucose and triglyceride levels (Rudic et al. 2004). To address the question of whether the metabolic defects are due to a loss of rhythmicity in the SCN or in the peripheral clocks, mice with tissue-specific deletion of *Bmal1* in the liver or pancreas have been generated. Even though these mice show normal locomotor activity, they display disturbances in the maintenance of blood glucose levels. In liver-specific *Bmal1* KO mice, the circadian expression of key metabolic genes, such as *glucose transporter 2* (*Glut2*), is abolished. This results in mice being hypoglycemic during the fasting phase of the feeding cycle (Lamia et al. 2008). Further illustrating the importance of peripheral circadian clocks, deletion of BMAL1 in the pancreas leads to diabetes (Marcheva et al. 2010; Sadacca et al. 2011). These mice display elevated blood glucose levels, impaired glucose tolerance, and decreased insulin secretion (for a review see Marcheva et al. 2013).

3.3.2 REV-ERBα

REV-ERBα was originally identified as a nuclear receptor that regulates lipid metabolism and adipogenesis (Fontaine et al. 2003). Thus, the role of *Rev-Erbα*

in controlling *Bmal1* expression—a function that provides robustness to circadian oscillations (Preitner et al. 2002)—established a critical link between the molecular machinery that regulates circadian oscillations and metabolism. Although *Rev-Erbα* $^{-/-}$ mice are not arrhythmic, the rhythmicity in their locomotor activity is altered (a shorter period length under constant light or constant dark conditions) (Preitner et al. 2002).

REV-ERBα appears to act downstream of PPARγ, a key regulator of fat metabolism and adipocyte differentiation (Fontaine et al. 2003). Genes involved in lipid metabolism in the liver also appear to be major targets of REV-ERBα. Depletion of REV-ERBα was shown to cause fatty liver phenotype, such as increased hepatic lipid and triglyceride content (Feng et al. 2011).

4 Conclusion

The importance of epigenetic control is becoming clear in the regulation of circadian rhythms. Current data suggests that many epigenetic regulators themselves are regulated in a circadian manner, at least in some tissues. The challenge ahead is to understand whether these epigenetic events follow a rhythmic pattern in tissues that are involved in diverse physiologies, such as process of learning and memory (e.g., hippocampus, cortex, and amygdala) and metabolism (e.g., liver, adipose tissue, kidney). As more data accumulates describing specific mechanistic roles of clock genes in regulating cellular proliferation and metabolic pathways, new therapeutic targets are emerging. As the pharma industry is converging on epigenetic regulators as promising targets for therapy, it is conceivable that drugs that modulate the clock function may result effective in specific strategies against certain types of cancer and metabolic disorders.

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Peripheral Circadian Oscillators in Mammals

Steven A. Brown and Abdelhalim Azzi

Abstract Although circadian rhythms in mammalian physiology and behavior are dependent upon a biological clock in the suprachiasmatic nuclei (SCN) of the hypothalamus, the molecular mechanism of this clock is in fact cell autonomous and conserved in nearly all cells of the body. Thus, the SCN serves in part as a "master clock," synchronizing "slave" clocks in peripheral tissues, and in part directly orchestrates circadian physiology. In this chapter, we first consider the detailed mechanism of peripheral clocks as compared to clocks in the SCN and how mechanistic differences facilitate their functions. Next, we discuss the different mechanisms by which peripheral tissues can be entrained to the SCN and to the environment. Finally, we look directly at how peripheral oscillators control circadian physiology in cells and tissues.

Keywords Feeding • Fibroblast • HPA axis • Temperature

1 Introduction: The Discovery of Peripheral Clocks

The basic unit of circadian timekeeping is the cell. Because clocks had been discovered in many unicellular organisms, it was obvious even half a century ago that individual cells *can* possess machinery to tell time. Nevertheless, in 1972, lesion studies identified a single tissue, the suprachiasmatic nuclei (SCN) of the hypothalamus, as necessary for circadian physiology and behavior in mammals (Stephan and Zucker 1972), and soon thereafter, central clock tissues or cells were also identified in birds (Takahashi and Menaker 1979), reptiles (Janik et al. 1990), and fruit flies (Liu et al. 1988). Therefore, most investigators twenty years

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ago imagined a centralized circadian timekeeping system through which signals from a master clock tissue orchestrated different diurnal processes in metazoans (Kawamura and Ibuka 1978).

The discovery of specific clock genes expressed in most cells created the possibility and the motivation to question this hypothesis. If clock function were based upon feedback loops of transcriptional repression (Hardin et al. 1990) and the genes and proteins involved in this mechanism were conserved in all metazoans and present in all tissues, then it would be possible to envision cell-autonomous clocks even in highly complex organisms. Indeed, in 1995, Welsh and colleagues showed that dispersed neurons of the suprachiasmatic nucleus each contained independently ticking clocks, as evinced by slightly different period lengths of spontaneous electrical activity. Timekeeping continued even when this electrical activity was blocked (Welsh et al. 1995). Similar clocks were also found in cultured retina (Tosini and Menaker 1996). Hence, in mammals as in bacteria, circadian timekeeping could be cell autonomous.

The finding of clock genes also permitted the invention of new technologies to probe clock function at a molecular level. By creating DNA "reporters" that use clock gene sequences to drive expression of bioluminescent or fluorescent proteins in individual cells, investigators could for the first time ask about clock gene function in different parts of an organism separately and noninvasively. Such a technology applied to fruit flies showed that different fly pieces contained autonomous clocks that functioned independently of "master clock" pacemaker neurons in the fly head (Plautz et al. 1997), and even cultured mammalian skin fibroblast cells contained autonomous clocks that tick in culture, completely independent of the SCN (Balsalobre et al. 1998).

The existence of peripheral clocks not only proved a significant boon to the understanding of clock mechanisms – now they could be studied in culture or in easily accessible tissues (Cuninkova and Brown 2008) – but also provoked a paradigm shift: maybe master clock tissues served not to send separate signals for different aspects of physiology but rather to synchronize peripheral clocks in other tissues, which in turn autonomously controlled circadian physiology.

The last decade has shown that both purely centralized and purely peripheral models are too simple. In reality, some aspects of mammalian physiology are controlled by peripheral clocks and others directly by central signals. Similarly, peripheral clocks sometimes accept signals from the SCN and sometimes take cues directly from their environment, and their entrainment has proven to be a web of both direct and indirect signals that can even vary from tissue to tissue. In this chapter, we shall begin by considering the molecular mechanisms of peripheral clocks and their similarities and differences to central clocks. Subsequently, we consider the mechanisms by which they are entrained and finally the complex physiology that they control in mammals.

2 Peripheral Clock Mechanisms

As a whole, the mechanism of circadian clocks in peripheral cells is remarkably similar to that of the "master" clock in SCN cells. For example, in humans, circadian period length measured in peripheral skin fibroblasts in vitro is directly

proportional to the circadian period of SCN-controlled behavior in the same subjects (Pagani et al. 2010). Moreover, analysis of peripheral and central clocks in mice deficient for individual clock proteins showed clearly that the broad outline of clock mechanism is the same in fibroblasts as in SCN (Yagita et al. 2001): feedback loops of transcription, translation, and posttranslational modification control most studied aspects of cellular circadian physiology.

As described in previous chapters, these loops are thought to be based upon a set of transcriptional activators (the CLOCK and BMAL1 proteins), which activate a set of repressor genes (the period loci *Per1-3* and cryptochrome loci *Cry1-2*), whose protein products repress their own transcription. In a separate loop, the nuclear receptor ROR and REV-ERB proteins activate and repress the *Bmal1* gene, respectively. Connecting these loops, the *Rev-Erbα* gene is itself regulated by CLOCK and BMAL1. (See Buhr and Takahashi 2013) for a detailed and referenced description of these molecular mechanisms.) In spite of this close overall similarity, on the level of gene expression, the cell-autonomous clocks ticking in each tissue have a slightly different set of core and associated clock loci directly involved in their timekeeping mechanism, and these differences have significant ramifications for the physiology that they direct.

2.1 Complements of Clock Genes and Proteins Vary from Tissue to Tissue

Although most identified "core clock genes" are present in most tissues, in some cases homologous genes assume different tissue-specific functions. For example, a deletion of one of the three mammalian *period* homologs, *Per3*, has only the subtlest of effects on the central clock mechanism (Shearman et al. 2000). However, some specific peripheral tissues like pituitary, liver, and aorta show a pronounced effect of *Per3* deletion on clock period length in tissue explants and clock phase in vivo (Pendergast et al. 2011). Therefore, it is likely that PER3 plays an important role in clock mechanism in some tissues but is redundant in others.

A similar functional overlap exists between CLOCK and its homolog NPAS2. In the SCN, loss of CLOCK protein is probably compensated by the presence of NPAS2, so that mice deficient for the *Clock* gene are behaviorally rhythmic (Debruyne et al. 2006), but in most peripheral tissues, CLOCK deletion leads to arhythmicity of circadian oscillators in tissue explants (DeBruyne et al. 2007a, 2007b) as well as in vivo (Dallmann et al. unpublished). In reverse, NPAS2 is believed to be important for the clock in the forebrain (Reick et al. 2001).

In addition, various auxiliary factors can play important tissue-specific roles in clock function. For example, oligophrenin 1 appears to regulate circadian oscillations in the hippocampus by interacting with REV-ERBα and modifying its transcriptional repression activity (Valnegri et al. 2011). Similarly, the two isoforms of AMP kinase (which are thought to phosphorylate CRY proteins

(Lamia et al. 2009)) have dramatic but tissue-specific effects upon circadian oscillator function (Um et al. 2011). Finally, a range of nuclear receptor proteins can interact with clock proteins such as REV-ERBα (itself a nuclear receptor) and PERs (Schmutz et al. 2010), and the tissue-specific distribution of such receptors likely leads to tissue-specific differences in circadian function (Teboul et al. 2009).

More broadly, both in vivo and in vitro, different mouse tissues show different circadian phases in tissue explants (Yamazaki et al. 2000; Yoo et al. 2004). While a portion of this variation is undoubtedly due to differences in entrainment signals, another portion is probably due to intrinsic variation in period from tissue to tissue – with shorter periods leading to earlier phases. For example, a five-hour phase difference is observable between liver and spleen, and nearly eight hours between liver and gonadal adipose tissue. Supporting a tissue-intrinsic mechanism for these phase differences, free-running period in tissue explants differed by 2–4 hours between liver and the other two tissues (Pendergast et al. 2012), again pointing to subtle tissue-specific differences in free-running clock mechanism. Intriguingly, testis is the only mammalian tissue that, so far, has not been shown to harbor a self-sustained clock (Alvarez et al. 2003).

These results demonstrate that each tissue can have its own complement of core clock genes that may vary in abundance or function. This sometimes subtle variation could lead to pronounced tissue specificity in clock-controlled output genes, as discussed later.

2.2 *Peripheral Tissues Lack Neuropeptidergic Signaling that Promotes Network Synchrony*

The second major difference between central and peripheral clocks relates to their network properties. Cultured fibroblasts and tissue explants from peripheral organs like liver, spleen, kidney, heart, and lung exhibit robust circadian oscillations in gene expression, at least initially (Yamazaki et al. 2000). However, all of these peripheral clocks have in common that their oscillations damp rapidly in culture. In contrast to peripheral tissues, SCN explants are capable of generating rhythmic gene expression and electrical activity for weeks or even years in culture. Interestingly, this damping has little to do with the cell-autonomous properties of peripheral and SCN cells. For example, cultured fibroblasts show persistent oscillations in culture that exceed the robustness of individual SCN neurons (Welsh et al. 2004). In fact, even though intact SCN explants show remarkably persistent oscillations, dispersed SCN neurons show very intermittent oscillations (Webb et al. 2009). The difference between SCN and periphery lies in coupling: whereas peripheral cells oscillate mostly independently of one another in vitro (Nagoshi et al. 2004; Welsh et al. 2004), SCN neurons possess specific mechanisms to maintain synchrony as a population and even appear to require them for stable oscillations.

Three different mechanisms appear to be used for coupling: synaptic potentials, electrical synapses, and neuropeptidergic signaling. The first two are common to most neurons: inhibition of voltage-dependent sodium channels (Welsh et al. 1995), GABAergic signaling (Albus et al. 2005), or gap junctions formed by connexins (Long et al. 2005; Shinohara et al. 2000) reduces the synchrony of SCN neuron populations in vitro. The third mechanism is more unique: neuropeptidergic coupling. Circadian secretion of vasoactive intestinal peptide (VIP) by a subset of SCN neurons is perceived as a paracrine timing cue by neighboring cells expressing its receptor, VPAC2. Loss of this coupling mechanism, either by ablation of VIP or of VPAC2, abolishes the circadian firing rhythm of a subset of SCN neurons, and mice harboring this mutation are therefore incapable of normal circadian rest/activity rhythms (Aton et al. 2005; Colwell et al. 2003). In total, it is likely that three neurotransmitter systems play overlapping roles in this coupling: primarily VIP, with contributions from arginine vasopressin (AVP) and gastrin releasing peptide (GRP) (Maywood et al. 2011). Other neurotransmitter systems may also play a role through tonic signaling. For example, the PAC1 receptor is normally involved in the response of the SCN to light, but deletion of the PAC1 receptor also changes circadian expression of VIP (Georg et al. 2007).

Although circadian peptidergic signaling is so far believed to be unique to the SCN, other mechanisms are certainly present in other tissues—e.g., sodium channels in heart or gap junctions in liver—and may be useful to achieve some degree of coupling. For example, in SCN-lesioned animals, individual organs still maintain some degree of circadian synchrony in clock gene expression, although this varies both among animals and among organs (Yoo et al. 2004). Nevertheless, it is universally accepted that this coupling is much less than in SCN. At a cellular level, there are two consequences of this lack. First, clock mechanisms in peripheral cells are more susceptible to mutation. For example, disruptions of individual nonessential clock genes have larger effects upon clock function in cultured fibroblasts than upon behavior in the same mice (Brown et al. 2005; Liu et al. 2007). This observation is clearly a consequence of greater coupling in SCN cells because larger effects can also be seen in dissociated SCN cells vs. intact slices (Liu et al. 2007). Secondly, the lesser coupling of peripheral cells permits greater phase shifting, making peripheral oscillators less "rigid." At least in vitro, this means that clocks from peripheral tissues (e.g., lung) can entrain to more extreme zeitgeber cycles, whereas the more rigid SCN clock will instead "free run" at its own intrinsic period (Abraham et al. 2010).

3 Entrainment of Peripheral Clocks

As mentioned in the previous paragraph, one consequence of mechanistic differences between oscillators in peripheral tissues and those in the SCN is variation in susceptibility to entrainment signals. Indeed, the most fundamental difference between central and peripheral oscillators lies in the signals to which they respond. A key characteristic of peripheral oscillators is their ability to respond

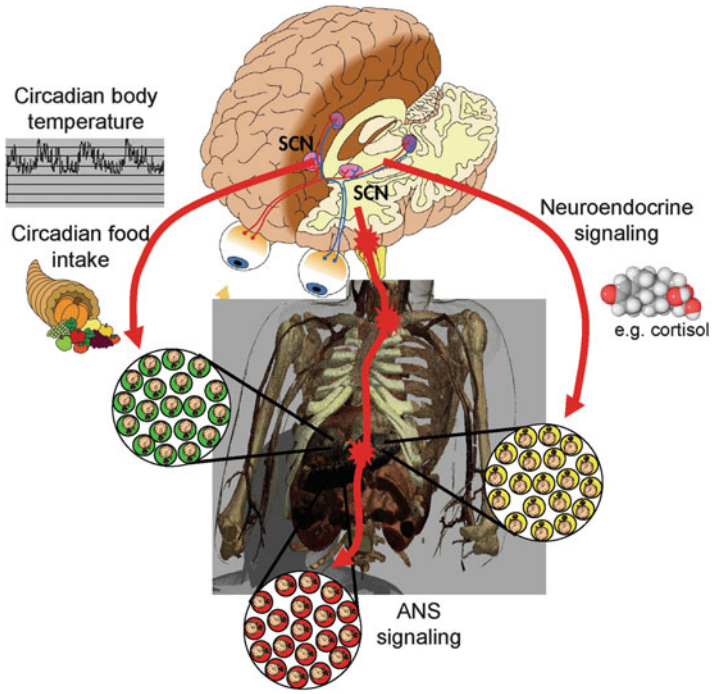

Fig. 1 Signals from SCN to peripheral oscillators. Synchronizing signals include direct nervous signals from the autonomous nervous system, neuroendocrine signals like glucocorticoids, and indirect signals such as circadian body temperature and food intake, which are both determined under normal circumstances by patterns of activity and rest (This diagram was adapted from original drawings by N. Roggli, as well as images from the Visible Human Project of the USNLM)

to SCN-driven timing signals and that of the "master clock" in the SCN is its blindness to these signals and instead its entrainment to a limited range of environmental stimuli. In general, whereas the SCN responds primarily to environmental light – a phenomenon described by Slat et al. (2013) and Roenneberg et al. (2013) – peripheral clocks are thought to respond to a complex and redundant combination of direct nervous stimuli, hormonal signals, and indirect activity-directed signals such as body temperature and the timing of food intake. These signals are described below and summarized in Fig. 1.

3.1 Entrainment by Direct Nervous Stimuli

SCN neurons project throughout the brain and, via their spontaneous circadian firing activity, are thought to provide signals for a wide variety of circadian behaviors. For example, projections to the subparaventricular zone (SPVZ) are responsible for circadian rhythms of locomotor activity via multiple hypothalamic arousal systems

(Abrahamson and Moore 2006). Similarly, reduced firing activity of the SCN during the late sleep phase directly affects osmoregulatory neurons that control vasopressin release and thereby suppress urination (Trudel and Bourque 2012). GABAergic input from the SCN to the paraventricular nucleus (PVN) controls circadian glucose production in the liver (Kalsbeek et al. 2004) and melatonin production in the pineal gland (Kalsbeek et al. 2000). An anatomically separate stimulatory output from the SCN is also necessary for correct circadian melatonin production (Perreau-Lenz et al. 2003). For the regulation of sleep and arousal, SCN projection to the locus coeruleus (LC) via the dorsomedial hypothalamus (DMH) is believed to play a central role, and LC neuronal activity displays a circadian firing rhythm (Aston-Jones et al. 2001). For the moment, although projections from the SCN to other brain regions directly regulate neural activity in target areas, it is unclear whether they also regulate cell-autonomous circadian clocks in target cells.

Beyond the brain, the autonomous nervous system plays a direct role in communicating circadian SCN timing signals to multiple tissues. For example, from the PVN, SCN signals travel via the autonomous nervous system to the liver to control glucose production (Kalsbeek et al. 2004). A multisynaptic autonomic nervous connection also exists between SCN and heart to regulate cardiac rate in circadian fashion (Scheer et al. 2001) and to the adrenal gland to regulate both circadian and light-dependent corticosterone production (Ishida et al. 2005). These examples are likely to represent only a small portion of physiology directly mediated by autonomous SCN connections: in total, sympathetic efferents have been documented for brown adipose tissue, thyroid gland, kidney, bladder, spleen, adrenal medulla, and adrenal cortex. Parasympathetic nervous system innervation of the thyroid, liver, pancreas, and submandibular gland has also been reported. Thus, some tissues are even innervated both sympathetically and parasympathetically by the SCN. Again, the functional implications of many of these connections are as yet uncertain (Bartness et al. 2001).

From the literature cited above, it is clear that at least some direct nervous efferents, both sympathetic and parasympathetic, can control circadian physiology. Based upon the analysis of clock gene expression in peripheral organs of hamsters whose two suprachiasmatic nuclei showed different phases, it is also clear that such signals can also play a role in the phase entrainment of peripheral clocks in some peripheral organs, like skeletal muscle, adrenal medulla, and lung, but not in others like liver or kidney (Mahoney et al. 2010). Given the ability of several neurotransmitter classes to act via pathways that phase-shift cellular clocks (e.g., cAMP and MAP kinase cascades), such control would not be surprising. Moreover, most of the direct nervous connections studied—either through hormones or effects upon behavior—can also influence peripheral clocks indirectly, as discussed next.

3.2 Entrainment by Peptides and Hormones

A second major path controlling circadian clocks is hormonal. Although nervous efferents from the SCN clearly play an important role, it has long been clear that this role is not essential, at least for the control of diurnal behavior. Lesion of the

SCN results in arrhythmic behavior, but implantation of fetal SCN tissue can rescue circadian locomotor activity, even when such an implant is encased in porous plastic (Silver et al. 1996). Therefore, diffusible factors from the SCN are capable of entraining circadian behavior. So far, two diffusible timing factors have been identified: transforming growth factor alpha (TGFα) (Kramer et al. 2001) and prokineticin 2 (PK2) (Cheng et al. 2002). These signaling proteins alter locomotor activity when injected chronically into the third ventricle, and both are secreted in circadian fashion by the SCN. While neither factor directly resets peripheral clocks, their control of activity provides indirect signals that do, as discussed below. Multiple other factors might also be important: recent advances in analytical technologies have enabled direct, high-resolution peptidomic profiles of rat SCN neurons, which produce a total of 102 endogenous peptides (Lee et al. 2010).

Another way by which the SCN entrains circadian physiology and gene expression in peripheral clocks is via the pituitary–adrenocortical axis, specifically via glucocorticoids, a class of steroid hormones that bind to the glucocorticoid receptor (GR). These hormones are secreted in daily fashion, and their receptors (GR) are expressed in most peripheral cell types, but not in SCN neurons. In addition to the critical role that glucocorticoids play in metabolism, it has been shown that in vitro and in vivo application of the glucocorticoid analog dexamethasone induces *Per1* expression in RAT1 fibroblasts and shifts or resets the phase of circadian gene expression in peripheral tissues but not SCN. Glucocorticoids are redundant with other timing signals because mice lacking GR in the liver still express genes in circadian manner in this organ (Balsalobre et al. 2000a).

Beyond glucocorticoids, at least in vitro, input to three other classes of signaling pathways has been identified as capable of independently phase-shifting peripheral circadian clocks: cAMP and MAP kinases, protein kinase C, and calcium signaling (Balsalobre et al. 2000b). Multiple signaling agents acting through these pathways have been shown to induce and synchronize circadian clocks in vitro, including endothelin-1 (Yagita et al. 2001), fibroblast growth factor, epidermal growth factor (Akashi and Nishida 2000), forskolin (Yagita and Okamura 2000), glucose (Hirota et al. 2002), and prostaglandin E2 (Tsuchiya et al. 2005). Based upon different phase shifting profiles, these various agents appear to intersect the known circadian clockwork in at least two different nodes, one showing rapid induction of the clock gene *Per1* and the other slow and weak induction of it (Izumo et al. 2006).

How this myriad of signals controls circadian phase in peripheral oscillators in vivo is until now unclear: only prostaglandin E2 and dexamethasone have been shown to shift circadian clocks acutely in peripheral organs when injected into mice (Balsalobre et al. 2000b; Tsuchiya et al. 2005), and all implicated pathways are essential for proper development, rendering conventional loss-of-function studies difficult. Nevertheless, in the case of glucocorticoid signaling, conditional and tissue-specific disruptions have allowed investigators to show unambiguously that glucocorticoid signaling plays an important role in the timing of circadian physiology, gene expression, and clock phase, especially in the liver (Kornmann et al. 2007; Reddy et al. 2007). Similar approaches with other signaling pathways should yield important information about roles of other hormone-dependent signaling cascades in peripheral circadian physiology.

3.3 Entrainment by Indirect Cues: Temperature and Feeding

In addition to direct cascades leading from the SCN to entrain peripheral clocks, there exist two important indirect cues that arise as a consequence of circadian behavior: temperature and food intake. Even in homeotherms such as mammals, circadian rhythms of activity and metabolism direct subtle fluctuations in body temperature (1–4 degrees Celsius, depending upon the organism). Both in cells and in living mammals, these rhythms are sufficient to entrain peripheral circadian oscillators (Abraham et al. 2010; Brown et al. 2002; Buhr et al. 2010), possibly via circadian oscillations in the activation of the same transcription factors that regulate the response of cells to acute heat shock (Reinke et al. 2008).

Similarly, patterns of feeding can directly entrain clocks in peripheral organs: inversion of the timing of food availability will inverse the timing of peripheral clocks, independently of the suprachiasmatic nucleus (Damiola et al. 2000; Stokkan et al. 2001). The speed as well as the degree of phase shift induced by inversed feeding varies among different organs. For example, mRNA of the clock gene *Dbp* examined in mice fed only during the light phase shows a strong temporal difference in liver, kidney, heart, and pancreas, whereas in mice fed during the dark phase, the accumulation of Dbp mRNA was around ZT14 to ZT18 in all analyzed tissues.

The mechanism by which peripheral oscillators can be entrained by food remains unclear. Since glucose itself can reset circadian clocks in cultured cells, it has been suggested that this simple food metabolite could play a role (Hirota et al. 2002). More broadly, circadian clock function is regulated in a variety of ways by cellular redox potential, which itself fluctuates via metabolism. The dimerization of CLOCK and BMAL1 and their binding to *cis-acting* DNA elements is itself regulated by redox potential, at least in vitro (Rutter et al. 2001), and the NAD +−dependent histone deacetylase SIRT1 directly interacts with the CLOCK: BMAL1 heterodimer to facilitate deacetylation and degradation of PER2 (Asher et al. 2008) and deacetylation of BMAL1 and local histones (Nakahata et al. 2008). At the same time, the NAD+−dependent ADP-ribosylate PARP1 interacts with CLOCK to ADP-ribosylate it and interfere with its binding, a process also important for correct entrainment to feeding (Asher et al. 2010). Another method of synchronizing circadian clocks to metabolism is probably mediated by cryptochrome clock proteins, which are phosphorylated and targeted for degradation by AMP-dependent kinase (AMPK), an enzyme regulated by cellular ATP/AMP balance (Lamia et al. 2009).

Other possible contributors to food-dependent entrainment of peripheral clocks are feeding-dependent hormones. Although glucocorticoids are obviously important to metabolic regulation, they appear to play no role. In fact, their signal opposes that of inversed feeding, and mice with tissue-specific loss of glucocorticoid receptor entrain much faster to changes in feeding schedules (Le Minh et al. 2001). By contrast, the hormone ghrelin might contribute to clock entrainment by feeding. Ghrelin is a 28-amino acid peptide produced mainly by P/D1 cells

covering the stomach and epsilon cells of the pancreas. It has also been reported that ghrelin levels exhibit a circadian rhythm and follow feeding schedules. Thus, it has been postulated that ghrelin-secreting cells are themselves entrained by feeding and then their hormonal signal serves as a messenger to other cells, both in the brain and in other peripheral tissues (LeSauter et al. 2009). Importantly, ghrelin can also modify SCN phase or its response to light both in vivo and in vitro, making it a candidate for broader modifications in circadian behavior in response to restricted feeding (Yannielli et al. 2007; Yi et al. 2008).

3.4 How the SCN Avoids Entraining Itself

The SCN sends a wide diversity of signals to entrain peripheral circadian physiology. However, at least theoretically, it is important that it remains insensitive to such signals. Otherwise, strong damping of oscillations would be predicted. Several biological mechanisms have been elucidated to achieve this end and render the SCN blind to the entrainment signals that it sends to peripheral tissues. For nervous signals, the problem is easily resolved: by definition, such signals are directional. For hormonal stimulation, the problem is more difficult because many hormones can cross the blood–brain barrier. Interestingly, however, the best-characterized hormone for entrainment of peripheral clocks, glucocorticoid hormone, has few or no receptors on SCN cells (Balsalobre et al. 2000a).

For indirect signals like temperature and food, the problem is even more complicated: heat shock factor, for example, is universally present in cells, as are sirtuins. In the case of temperature variation, the SCN is clearly not entrained like peripheral cells: inversing circadian body temperature fluctuations in mice by environmental temperature cycles will inverse circadian gene expression in peripheral cells (including non-SCN brain regions, in spite of innervation from the SCN). The SCN itself, however, is unaffected (Brown et al. 2002). Exactly why the SCN is resistant to such entrainment signals is an important question that recent studies have helped to clarify. Interestingly, the "temperature resistance" of the SCN is a network property and not a cell-autonomous one—i.e., SCN neurons in an intact network are insensitive to temperature signals, but dissociated SCN neurons are not (Buhr et al. 2010). The most likely explanation for this phenomenon is that SCN network properties render its clock more "rigid," which would permit entrainment to environmental signals within only a narrow range. As a practical result, sudden dramatic changes in period or phase of temperature signals would be ignored (Abraham et al. 2010). The latter model could also explain failure to entrain to sudden changes in feeding signals as well: for mice subjected to inverted feeding cycles, the SCN remains unshifted even as peripheral clocks change up to 180 degrees in phase (Damiola et al. 2000; Stokkan et al. 2001). In the case of the latter model, however, a specific exception would have to be made for light-dependent phase shifting: for mice subjected to sudden "jet lag" with shifts in light and via activity rhythms food and temperature cycles also, different organs shift at different

rates, but the SCN is among the most rapid to adopt the new phase (Davidson et al. 2008; Yamazaki et al. 2000).

4 Physiological Control by Peripheral Circadian Clocks

A large number of physiological processes are under circadian control. These include xenobiotic detoxification, lipid metabolism, renal plasma flow and urine production, cardiovascular parameters such as blood pressure and heart beat rates, and even many aspects of immune function (Gachon et al. 2004). The cell-autonomous nature of the circadian clock, coupled with its hierarchical entrainment structure in mammals, would suggest that circadian physiology in peripheral tissues is largely controlled by peripheral oscillators. In fact, this statement is only partially true. Certainly, many aspects of diurnal physiology in peripheral tissues are directly dependent upon circadian clocks in these tissues. Other aspects, however, are controlled by circadian autonomous nervous or hormonal signals indirectly originating from the SCN.

4.1 Cell-Autonomous Circadian Physiology

As described above and in previous chapters, the canonical circadian clock mechanism is controlled by transcriptional feedback loops in which clock proteins bind to *cis*-acting DNA elements to activate or repress the expression of other clock proteins. Interestingly, however, these same elements are present throughout the genome and regulate clock-controlled genes as well (Ripperger et al. 2000). Therefore, they probably serve as one of the principal conduits by which peripheral circadian physiology is directed. Such rhythmic transcriptional control is believed to be generated through three principal binding motifs in promoter regions: E-boxes, D-boxes, and Rev-Erbα/ROR-A response elements (RREs) (Ueda et al. 2005; Minami et al. 2013). Various combinations of these elements are capable of generating a wide variety of phase profiles. In total, about ten percent of transcripts in all peripheral tissues are regulated in circadian fashion (Panda et al. 2002; Storch et al. 2002; Reddy 2013).

Recently, genome-wide technologies—ChIPseq to identify binding sites for particular proteins on a genomic scale, RNAseq to identify sequences present in all transcripts, etc.—have dramatically increased our knowledge of how clock factors control gene expression in peripheral tissues and of which pathways are controlled (Reddy 2013). For example, genome-wide analyses of binding targets of BMAL1 (Hatanaka et al. 2010; Rey et al. 2011) and multiple other circadian clock factors in liver (Koike et al. 2012) have clarified not only which pathways are controlled (particularly carbohydrate and lipid metabolism) but also how different regulatory elements contribute to this regulation. Similar profiling of REV-ERBα

and REV-ERBβ targets has shown liver regulation of both core circadian clock and metabolic networks by both proteins (Cho et al. 2012).

In the liver, whose circadian physiology has been particularly well studied, one example of peripheral clock-directed transcriptional control is furnished by xenobiotic metabolism pathways. Here, circadian transcription of PAR-B-ZIP (proline- and acidic amino acid-rich basic leucine zipper) transcription factors like *Dbp* (D-element binding protein) is controlled by the clock proteins CLOCK and BMAL1 via *cis*-acting E-box elements (Ripperger et al. 2000). PAR-B-ZIP factors bind to D elements in the promoter of the constitutive androstane receptor (CAR) gene, which in turn controls circadian expression of many cytochrome P450 isoforms that directly regulate metabolism of a wide variety of xenobiotics (Gachon and Firsov 2010; Gachon et al. 2006). This cascade of circadian transcription factors is diagrammed in Fig. 2. The same three PAR-B-ZIP factors also play a key role in directing circadian lipid metabolism by controlling expression of the *PPARα* (peroxisome proliferator-activated receptor alpha) gene (Gachon et al. 2011). Liver glucose metabolism is also strongly regulated by the cell-intrinsic liver circadian clock. In fact, peripheral clock-regulated hepatic glucose export probably counterbalances feeding-driven rhythms of daily glucose ingestion in order to maintain relative homeostasis (Lamia et al. 2008). Although the control mechanisms described above highlight transcriptional mechanisms based upon repression and initiation of transcriptional initiation, an increasing number of studies suggest that other later steps in transcription (Koike et al. 2012), including RNA export or stability (Morf et al. 2012), transcriptional termination (Padmanabhan et al. 2012), and splicing (McGlincy et al. 2012), also play important roles. Since the percentage of circadian proteins in liver is greater than the number of circadian transcripts (Reddy et al. 2006), it is likely that entirely posttranscriptional circadian regulatory mechanisms are also operative.

Although these studies were done mostly in liver, peripheral circadian clocks also play a strong role in many other organs. For example, the strong circadian rhythmicity of renal function has long been known (Minors and Waterhouse 1982). However, core clock transcripts like *Clock, Bmal1, Npas2, Per1-3,* and *Cry1-2* are expressed in the distal nephron with robust oscillations, and mice lacking either CLOCK or PAR-B-ZIP factors show significant changes in renal expression of key regulators of water and sodium balance, as well as changes in sodium excretion itself (Zuber et al. 2009). Therefore, kidney-intrinsic circadian oscillators are likely to play a key role in physiological regulation by this tissue. Likewise, circadian clocks in macrophages (Keller et al. 2009) and T cells (Fortier et al. 2011) govern inflammatory immune responses, and the clock protein REV-ERBα appears to play a specific role in selectively regulating inflammatory cytokines (Gibbs et al. 2012).

In other tissues, the retinal circadian clock is essential to circadian oscillations of light response in the inner retina (Storch et al. 2007). Moreover, arterial transplants from animals lacking circadian clocks develop atherosclerosis in transplanted blood vessels, proving a role for autonomous circadian clocks here as well (Cheng et al. 2011). Circadian clock ablation in pancreatic islets results in diabetes due to defects in coupling of beta cell stimulus to insulin secretion

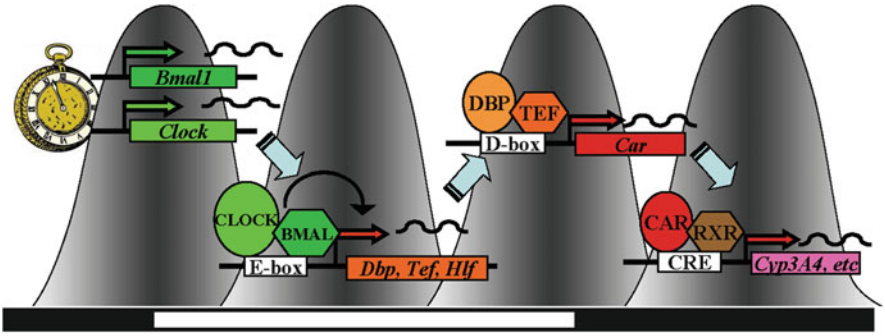

Fig. 2 Circadian transcription factor cascades determining xenobiotic metabolism in the liver. The transcriptional activators CLOCK and BMAL1, parts of the fundamental mechanism of the circadian oscillator, activate transcription of genes encoding the PAR-B-ZIP transcription factors DBP, TEF, and HLF. These proteins in turn activate transcription of the constitutive androstane receptor (CAR). (For clarity, only DBP and TEF are pictured.) The CAR protein then activates transcription of cytochrome P450 loci, either alone or as a dimer with RXR, the retinoid X receptor

(Marcheva et al. 2010). In cardiac tissues, peripheral clocks control expression of multiple kinases and ion channels, and cardiac clock mutation changes physical activity (Ko et al. 2011) and cardiac triglyceride metabolism (Tsai et al. 2010). Mutation of clocks in circulatory epithelium eliminates circadian rhythms in thrombogenesis (Westgate et al. 2008). Circadian transcriptome analyses of skeletal muscle and adipocyte tissues in tissue-specific clock-deleted animals show the regulation of at least 400 genes by muscle cell clocks and 660 by adipocyte clocks (Bray and Young 2009), suggesting that considerable circadian physiology in these tissues is peripherally regulated. Similarly, direct clock control of NAD+ salvage also implies that regulation of cellular metabolism is peripherally controlled (Nakahata et al. 2009). Finally, circadian clocks in adrenal tissue are essential for circadian production of glucocorticoids (Son et al. 2008), and clocks in target tissues possibly even control circadian glucocorticoid receptor expression (Charmandari et al. 2011). Similarly, circadian clock control of adrenal aldosterone production via the enzyme Hsd3b6 is an important regulator of blood pressure (Doi et al. 2010). For both of these hormones, their circadian biosynthesis is under control of adrenal circadian clocks, even if stimulation of the adrenal gland is sympathetically driven.

4.2 Direct Endocrine Control of Circadian Physiology

Although a considerable amount of circadian physiology is directed by peripheral clocks, another portion is not. A large number of circadian endocrine factors are able to directly elicit circadian physiological responses without contributions from peripheral clocks in target tissues. For example, tissue-specific disruption of

circadian clock function in liver and in other tissues has revealed that a portion of circadian gene expression is also systemically driven by neuroendocrine signals, most notably glucocorticoids. Disruption of liver clocks by interfering with *Bmal1* expression in vivo revealed 31 genes whose expression was still circadian (Kornmann et al. 2007). Comparable results have been seen in other tissues like muscle, heart, and fat (Bray and Young 2009). Similarly, glucocorticoid signaling is not only able to synchronize peripheral circadian oscillators (Balsalobre et al. 2000a), but it can also independently control 60% of the circadian transcriptome (Reddy et al. 2007). Interestingly, this control appears to be modulated by a direct interaction between glucocorticoid receptors and the cryptochrome clock proteins (Lamia et al. 2011). Other nuclear-receptor-coordinated physiology may also be modulated by direct interactions with clock proteins: PER2 has been shown to interact with PPARα and REV-ERBα (Schmutz et al. 2010). In the brain, direct interactions between REV-ERBα and oligophrenin 1 appear to play an important role in hippocampal circadian clocks and affect localization of REV-ERBα (supposedly a nuclear transcription factor) to synapses (Valnegri et al. 2011).

Circadian activity of the hypothalamic–pituitary–adrenal (HPA) axis is only one aspect of endocrine control of peripheral circadian physiology. A second example of endocrine regulation is the hormone melatonin, which exerts diverse circadian effects upon sleep and inflammation (Hardeland et al. 2011). Because so many endocrine factors are secreted in circadian fashion, numerous other examples exist, ranging from immune cytokines like TNFα to growth hormone and gonadal steroids like testosterone (Urbanski 2011). The circadian physiology that they control is considered in more detail (Kalsbeek and Fliers 2013).

4.3 Indirect Control of Circadian Physiology

Through its regulation of activity cycles and feeding, the SCN can not only send endocrine signals that regulate peripheral circadian clocks but also directly control circadian physiology. For example, in the mouse liver, only a small proportion of transcripts displayed circadian expression patterns in the absence of food, and conversely, temporally restricted feeding could restore circadian transcription of a sizable fraction of the circadian transcriptome even in the absence of functional liver clocks (Vollmers et al. 2009). Similarly, of 2,032 cortical transcripts under circadian control, only 391 remained rhythmic during sleep deprivation (Maret et al. 2007), thereby implying an essential contribution of rest–activity rhythms to circadian physiology and gene expression, at least in some tissues. Another recent study demonstrated how temperature fluctuations could drive circadian expression of some factors like cold-induced RNA-binding protein (CIRP) independently of the core circadian clock, reinforcing its function (Morf et al. 2012). Altogether, the exact contributions of these indirect cues in circadian physiology remain an exciting new aspect of clocks where tissue specificity could play an important role.

4.4 Circadian Physiology Controlled by Noncanonical Clocks

Most circadian physiology is controlled by the circadian clock mechanisms described above, based upon feedback loops of transcription and translation. Very recently, however, another independent circadian mechanism was elucidated in red blood cells, which lack nuclei and therefore transcription. Although the mechanism of this clock remains entirely unknown, it is able to direct circadian oscillations of oxidation and reduction in both heme-containing proteins and peroxiredoxins, a highly conserved family of scavengers of peroxide produced by respiration (O'Neill and Reddy 2010). This clock mechanism appears to be independent of the known repertoire of clock proteins, and the range of physiology that it controls remains a mystery (for a review, see O'Neill et al. 2013).

5 Summary

Certainly, the discovery of peripheral oscillators in mammals qualifies as one of the major discoveries in circadian biology during the past twenty years. Through the vast amount of circadian biology that they control, these clocks doubtlessly play an important role in diurnal physiology, and specific disruption of clocks in peripheral tissues of laboratory mice can create a wide range of pathologies (Marcheva et al. 2013) – diabetes (Marcheva et al. 2010), atherosclerosis (Cheng et al. 2011), glucose intolerance (Lamia et al. 2008), and defects in renal and cardiac function (Ko et al. 2011; Zuber et al. 2009).

More important for human pathophysiology, because of the complex web of direct and indirect signals by which peripheral clocks are synchronized, it is likely that additional pathophysiology results from desynchrony between peripheral and central oscillators. In several studied instances, complex interactions between central clocks and peripheral ones maintain critical homeostasis – for example, in the case of glucose and insulin (Lamia et al. 2008; Marcheva et al. 2010). Since jet lag and shift work result in differential rates of clock adjustment in different tissues (Davidson et al. 2008), it is probable that some of the adverse pathologies associated with these conditions both in the laboratory and in the real world, such as metabolic syndrome (De Bacquer et al. 2009) and immune dysfunction (Castanon-Cervantes et al. 2010), could arise from conflict between peripheral and central clocks rather than from adverse effects of circadian phase shift per se. In this case, creative manipulation of peripheral clocks by synchronizing cues could provide possible therapeutic benefits. For example, reinforcement of circadian timing in peripheral tissues by meal timing has been shown to inhibit cancer growth by 40% in mice, irrespective of caloric intake (Li et al. 2010).

In this review, we have tried to separate and enumerate the various different mechanisms and entrainment signals for peripheral circadian clocks, as well as the physiology that they control. The resulting picture that emerges, though complex, is

likely far too simple. In reality, it is likely that clocks in different tissues interact in many different layers. For example, as explained above for nuclear-receptor-mediated physiology, many NR ligands are expressed in circadian fashion via circadian neuroendocrine control by the autonomous nervous system, but the synthesis of these steroid hormones depends upon autonomous circadian clocks in endocrine tissues. Circadian oscillations in hormone abundance program a circadian physiological response in target tissues, but clock components in these tissues then provide a further layer of circadian regulation. The physiological consequences of such networks are not yet fully understood but will doubtlessly furnish fascinating and medically relevant subjects of investigation in the future.

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Cellular Mechanisms of Circadian Pacemaking: Beyond Transcriptional Loops

John S. O'Neill, Elizabeth S. Maywood, and Michael H. Hastings

Abstract Circadian clocks drive the daily rhythms in our physiology and behaviour that adapt us to the 24-h solar and social worlds. Because they impinge upon every facet of metabolism, their acute or chronic disruption compromises performance (both physical and mental) and systemic health, respectively. Equally, the presence of such rhythms has significant implications for pharmacological dynamics and efficacy, because the fate of a drug and the state of its therapeutic target will vary as a function of time of day. Improved understanding of the cellular and molecular biology of circadian clocks therefore offers novel approaches for therapeutic development, for both clock-related and other conditions. At the cellular level, circadian clocks are pivoted around a transcriptional/post-translational delayed feedback loop (TTFL) in which the activation of *Period* and *Cryptochrome* genes is negatively regulated by their cognate protein products. Synchrony between these, literally countless, cellular clocks across the organism is maintained by the principal circadian pacemaker, the suprachiasmatic nucleus (SCN) of the hypothalamus. Notwithstanding the success of the TTFL model, a diverse range of experimental studies has shown that it is insufficient to account for all properties of cellular pacemaking. Most strikingly, circadian cycles of metabolic status can continue in human red blood cells, devoid of nuclei and thus incompetent to sustain a TTFL. Recent interest has therefore focused on the role of oscillatory cytosolic mechanisms as partners to the TTFL. In particular, cAMP- and Ca^{2+}-dependent signalling are important components of the clock, whilst timekeeping activity is also sensitive to a series of highly conserved kinases and phosphatases. This has led

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to the view that the 'proto-clock' may have been a cytosolic, metabolic oscillation onto which evolution has bolted TTFLs to provide robustness and amplify circadian outputs in the form of rhythmic gene expression. This evolutionary ascent of the clock has culminated in the SCN, a true pacemaker to the innumerable clock cells distributed across the body. On the basis of findings from our own and other laboratories, we propose a model of the SCN pacemaker that synthesises the themes of TTFLs, intracellular signalling, metabolic flux and interneuronal coupling that can account for its unique circadian properties and pre-eminence.

Keywords Intracellular • Circadian rhythms • Signal transduction • Metabolic regulation • SCN • Post-translational • Cytoscillator

1 Circadian Rhythms in Health and Disease, In Vivo and In Vitro

Circadian rhythms are biological oscillations with periods of approximately 1 day. They are manifest in the temporal organisation of behavioural, physiological, cellular and neuronal processes—influencing phenomena as diverse as sleep/wake cycles, glucose homeostasis, innate immunity and cell division. Because this endogenous timekeeping interacts with myriad biological systems, circadian disruption has significant impacts upon human health and the diseased state; e.g. whilst acute clock disruption can result in the short-term side effects known as jet lag, long-term shift workers (~15 % workforce in developed nations) suffer from chronic circadian dysregulation associated with an increased susceptibility to cardiovascular disease, type II diabetes and various cancers (Reddy and O'Neill 2010). In addition, many identified 'clock genes' could equally well be described as tumour suppressors, had they been first studied by oncologists, since their genetic lesion can lead to mis-regulation of both the cell and circadian cycle (Reddy et al. 2005). Put simply, because bodily processes change dramatically and predictably between day and night, there exists clear translational potential in elucidating the mechanistic basis for circadian timekeeping from the perspectives of novel therapeutic targets and therapeutic efficacy.

1.1 Why Be Rhythmic?

The capacity to anticipate temporal environmental cycles, brought about by the Earth's daily rotation, is thought to have conferred a constant selective pressure over evolutionary timescales such that, essentially, all eukaryotes and many prokaryotes exhibit intrinsic circadian timekeeping (Roenneberg and Merrow 2002). Whether or not circadian rhythms arose divergently, or several times, and converged across the different kingdoms of life is not presently clear. Certainly, however, there appear to be advantages associated with the temporal organisation of

physiology and metabolism, as evidenced by the yeast metabolic oscillation which separates in time biochemically incompatible catabolic and anabolic phases (Robertson et al. 2008). A well-known mammalian circadian equivalent is the clock-driven up-regulation of gluconeogenic transcripts in hepatocytes in anticipation of the nightly fast, regardless of feeding history (Akhtar et al. 2002). Whilst modern humans attempt to populate a 24-h society, our late Pleistocene genome still encodes the clock of a diurnal hunter-gatherer, resulting in hyperglycaemia and increased risk of the metabolic syndrome for those that indulge in midnight feasting. Even fruit flies maintained for 1,000 generations in constant darkness continued to be rhythmic demonstrating how deeply circadian rhythms are hardwired into cellular metabolism. Within this vein, the observation that populations of blind cavefish (*Phreatichthys andruzzii*) which have existed in constant darkness for ~2 million years have extremely long periods [nearly 2 days (Cavallari et al. 2011)] suggests that whilst rhythms are not readily dispensable, they can adapt under appropriate selection.

1.2 Pharmaceutically Relevant Circadian Principles

In mammals, daily timekeeping is now well established as a cell-intrinsic phenomenon, being observed in cultured cells and tissues over many days, in vitro. In spite of this, the language used to describe biological clocks was developed decades ago, when behavioural rhythms in experimental organisms were the most commonly studied output. As such, several criteria need to be defined with reference to drug action. First, a circadian rhythm is one exhibiting a period of approximately 24 h under constant conditions, i.e. in the absence of external time cues. Each point on the cycle can be assigned a unique phase, commonly expressed as hours of circadian time. Recent widespread application of genetically encoded real-time reporters has identified a small number of compounds that significantly and dose-dependently shift the cycle to a new phase and/or shorten or lengthen free-running period in cultured tissues and cells. Of these, few have been tested for their effect upon behavioural rhythms in mice, in vivo, but it is interesting to note that some of the earliest studies upon circadian rhythms were investigations into the period increase elicited by simple inorganic compounds, e.g. lithium salts and heavy water (Engelmann et al. 1976; Pittendrigh et al. 1973) (Fig. 1).

The biological clock is not a disembodied timer, but responds appropriately to its environment, and thereby allows organisms to cope with the varying day length and light quality associated with time of day, seasons and the weather. Relevant external cues entrain the phase of the oscillation, so that in nature the internal cycle is subtly reset each day (e.g. by dawn and dusk illumination, time of feeding, temperature cycles, etc.) in order to resonate with external solar cycles. For humans, light is the strongest entraining stimulus (or Zeitgeber), with intrinsic circadian phase being able to advance or delay by up to 60 min per day when light is experienced during late or early night, respectively (Skene and Arendt 2006). By exposing test subjects or model organisms to light pulses around the circadian cycle, a phase response curve may be described (Johnson 1999). This is relevant because similar

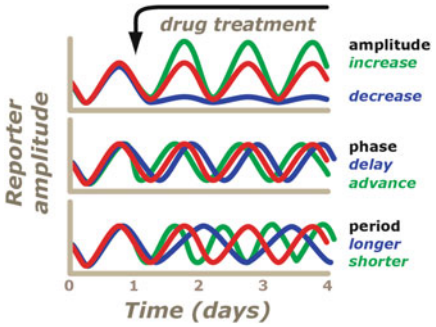

Fig. 1 Schematic of how drug treatment may affect circadian timekeeping. Using an appropriate reporter, drug treatment (*blue*, *green*) can elicit differential effects upon the properties of circadian rhythms (period, phase, amplitude) that persist in organisms and cultured cells (*red*, untreated). An appropriate reporter might include behavioural activity, metabolite concentration, gene expression, bioluminescence, fluorescence, etc.

phase-dependent shifts may be evoked in cultured tissues (Fig. 1), and animal behavioural rhythms, by pulsed drug application at particular circadian phases. Such experiments provide insight into the mechanisms that facilitate phase shifts, in vivo, as well as offering the potential for coordinated pharmacological manipulation of human rhythms in order to reduce the adverse long-term health effects that result from circadian misalignment with the external world. When organisms are under entrainment such as light/dark cycles, external time is reported with respect to the Zeitgeber time, ZT, such that ZT0 = dawn, ZT12 = dusk. Under constant conditions, circadian time, CT, is used and refers to endogenous timekeeping, i.e. CT0 corresponds to the phase that diurnal animals become active, whereas CT12 represents onset of activity in nocturnal species. These terms remain in use, although recently alternative models have been proposed (Roenneberg 2010).

An additional feature common to all circadian rhythms is that they are temperature compensated ($Q_{10} \sim 1$), the adaptability of which is intuitive since a clock that ran faster on warm days would be of little utility but unusual since most biological and chemical reactions double in rate for every 10 °C temperature increase. In other contexts, such compensation can result as an emergent network property or intramolecularly but generally involves mutually antagonistic processes that are each temperature dependent (Ruoff et al. 2007). Pharmacological manipulation of temperature compensation has been reported (Dibner et al. 2009), but it is the canonical clock property that seems least well understood in terms of mechanism.

2 Chronopharmacology

Over the last half century, models for the basis of rhythmicity have cycled from the biochemical to the electrophysiological, to the genetic and back (Edmunds 1983; King and Takahashi 2000; Njus et al. 1976). What is uncontested is that to produce

any oscillation, delayed negative feedback is required. For its amplitude not to damp out, some positive feedforward is also needed (Lenz and Sogaard-Andersen 2011). Regardless of timekeeping mechanism, therefore, and whether the application is medicinal or scientific, the action of any drug upon a biological target could be considered as an interaction between its pharmacology and biological timing, since the cell exists in a state of cyclical flux that affects many aspects of gene expression, macromolecular turnover and metabolism. 'Chronopharmacology' might be considered to consist of the following three separable, but related factors [which are discussed in greater detail in Ortiz-Tudela et al. (2013), Antoch and Kondratov (2013), and Musiek and Fitzgerald (2013)].

2.1 Chronopharmacokinetics

Many xenobiotic uptake, detoxification and clearance pathways are circadian regulated, meaning that the absorption, distribution, metabolism and elimination of drugs and their secondary metabolites, as well as accompanying side effects, may be affected by the circadian phase of administration. For example, evening dosing resulted in the lowest observed toxicities for NSAIDs in osteoarthritic patients (Levi and Schibler 2007).

2.2 Chronopharmacodynamics

If the concentration or activity of a drug target is circadian regulated (e.g. enzyme activities can be modulated at the level of transcription/translation, post-translational modification or spatial localisation, or secretion if extracellular), then this can be expected to have concomitant effects upon drug efficacy. For example, 'statins' have been known for years to be most efficacious when administered during subjective night (Muck et al. 2000), since the pharmacodynamics vary over the circadian cycle.

2.3 Chronoactivity

A relatively small number of drugs have been shown to affect cellular rhythms, in vitro, presumably by directly or indirectly modulating the activity of cellular components that play a role in timekeeping or pathways of entrainment. Whilst being useful as research tools, understanding the circadian action of such drugs in vivo is particularly complex, since tissues may respond differently and effects upon intrinsic timekeeping might be undesirable. For example, many aerosolised asthma medications contain glucocorticoids, but glucocorticoids have also been

shown to effect phase resetting in several tissues (Reddy et al. 2007). Thus, whilst their use may be life-saving in the short term, it is conceivable that some adverse side effects associated with their long-term use may be related to internal desynchronisation. In contrast, several psychiatric conditions are associated with disordered sleep/wake cycles, i.e. poorly organised behavioural rhythms. In the case of schizophrenics, a commonly prescribed mood stabiliser is lithium salts. Therapeutic doses (1–2 mM serum concentration) are around the level where a lengthening of free-running period may be observed in experimental organisms, and it has been proposed by several that the action of lithium on the biological clock may contribute to its positive treatment outcomes (Schulz and Steimer 2009).

2.4 Clinical Relevance

Clearly, in light of the massive undertaking represented by a modern clinical trial, it would be prohibitively expensive to investigate systematically all chronopharmacological aspects of a new or existing drug. Indeed, many initial observations were made anecdotally (Muck et al. 2000). The potential medical benefits offered by chronopharmacology, such as increased efficacy or reduced side effects through timing of treatment, are sufficient, however, to ensure that such factors will not be neglected indefinitely (Minami et al. 2009). The most cost-effective solution will surely be to reduce the complexity of the problem by approaching it in the light of existing knowledge and testing predictions in cellular and animal models first, because of the profound conservation of circadian principles between cells, tissues and species. As such, in the rest of this chapter, the state of existing knowledge about timekeeping mechanisms will be discussed. Particular emphasis will be placed upon various non-transcriptional mechanisms, since many of these are thought essential for circadian timekeeping and, more importantly, are likely to be more amenable to pharmacological manipulation than transcription.

3 Models and Mechanisms of Circadian Timekeeping

3.1 The SCN: First Amongst Equals

The suprachiasmatic nucleus (SCN) of the hypothalamus was long thought the centre of mammalian timekeeping, since its surgical ablation in rodents abolishes circadian rhythms in behaviour, body temperature and the secretion of endocrine factors such as melatonin and cortisol (Welsh et al. 2010). Moreover, SCN-ablated rodents receiving a surgical graft of foetal SCN tissue take on the circadian behavioural phenotype of the donor strain (King et al. 2003; Ralph et al. 1990). Recently, however, it has become apparent that circadian rhythms are a property

inherent to most, if not all, mammalian cells—being observed to persist throughout the body in vivo and in isolated tissues/cells for many days, in vitro (Welsh et al. 2004; Yoo et al. 2004). 10–20 % of mammalian genes are expressed rhythmically in one or more tissues (Reddy 2013), although specific 'clock-controlled genes' (CCGs) vary between tissues, appropriately to organ function (Deery et al. 2009; Doherty and Kay 2010; Reddy et al. 2006). The observation that some genes are expressed rhythmically has enabled the widespread application of real-time bioluminescent reporters for cellular rhythms, whereby firefly luciferase fusions with relevant genomic sequences enable non-invasive, long-term recordings of rhythmic bioluminescence to be made (Yamaguchi et al. 2000). Using reporters such as the PERIOD2::LUCIFERASE (PER2::LUC) knock-in mouse has enabled single-cell time-lapse imaging of molecular circadian rhythms in culture. These revealed that the accuracy and robustness of timekeeping in dissociated SCN neurons, and cultured fibroblasts, are poor compared with rhythms from intact SCN slices or whole animals [cycle-to-cycle period variation of ~2 %, 3 % and 9 % in mice, slices and neurons, respectively (Herzog et al. 2004)]. Thus, whilst circadian rhythms are a cellular phenomenon, clearly there are emergent network properties at higher levels of biological scale that make the whole greater than the sum of its parts.

Our view of the SCN has therefore evolved to become that of the primary locus for coordinating rhythms generated by innumerable subordinate cellular clocks distributed throughout the rest of the body. Comprising around 10–20,000 neurons, the SCN employs both humoral factors and axonal projections to other brain regions to maintain stable phase relationships between peripheral tissues (Welsh et al. 2010). Sited above the optic chiasm, a proportion of SCN neurons have excitatory glutamatergic innervations from the retinohypothalamic tract, receiving photic cues from both image- and non-image-forming photoreceptor cells, as well as non-photic signals from the brainstem (5HT, NPY) (Brown and Piggins 2007; Leak et al. 1999). Concomitant with biological function, therefore, the SCN is a heterogeneous assemblage of cells that integrates multiple inputs to maintain its phase of oscillation and in turn populates a diversity of output pathways to convey temporal cues appropriate to each target site (see also Slat et al. 2013). This complex cellular heterogeneity and circuit structure enables additional systems-level functionality, such as encoding of day length, to emerge (VanderLeest et al. 2007).

3.2 Level of Abstraction

To understand any biological system, it is helpful to reduce its complexity to the simplest level where the phenotype of interest may be observed. In circadian rhythms, traits such as free-running period of behavioural activity (under constant conditions), or the relative phases of gene expression, are used as proxies for timekeeping. This can cause problems, however, since animals which are behaviourally arrhythmic cannot be assumed to be deficient in cellular timekeeping, i.e. mice with no legs are also arrhythmic when assayed with running wheels.

Similarly, circadian rhythms in mammals should not really be studied at levels of scale below cellular until and unless the oscillation can be reconstituted biochemically in vitro (as has been demonstrated for the cyanobacterial oscillator, where a mixture of three Kai proteins plus ATP exhibit circadian cycles of autophosphorylation).

Since the correct level of biological abstraction at which to study circadian timekeeping is probably the cell (Noble 2008), it may be puzzling to scientists outside the circadian field that mechanisms of timekeeping within the SCN remain the subject of such intense scrutiny. Other than the momentum that gathers around any successful experimental model, this can be explained in several ways. First, the SCN is bona fide master clock—without it experimental rodents become behaviourally arrhythmic, and whilst timekeeping in peripheral tissues continues, they become desynchronised one from another and free-run with their own intrinsic period (Tahara et al. 2012). Therefore, we need to understand the SCN in order to place timekeeping within a physiological context. Second, unlike many neuronal cultures, organotypic SCN slices remain viable for many months in vitro and maintain many of their in vivo circadian properties, e.g. robustness, accuracy and interneuronal coupling. In addition, the SCN is amenable to media changes without perturbing the ongoing oscillation, and its circadian period is reflective of its genetic background. Finally, although the SCN is a highly specialised timekeeping organ, it appears to use several timekeeping mechanisms which are general to mammalian cells but with higher amplitude and are therefore more readily detectable.

3.3 The State of the Art: Circadian Timing by Transcriptional Feedback in Mammals

Based on both forward and reverse genetics, recent models of cellular timekeeping have focused on transcriptional/translational feedback mechanisms whereby positive activators (e.g. BMAL1 & CLOCK) bind to commonly occurring regulatory promoter elements (e.g. E-boxes) of many circadian-regulated genes, including those that encode transcriptional 'clock gene' repressors, e.g. PERIOD1/2 (PER1/2) and CRYPTOCHROME1/2 (CRY1/2), facilitating transcriptional activation around anticipated dawn (CT0). The repressor proteins are processed post-translationally, eventually accumulating to form complexes later in the day, prior to nuclear entry around anticipated dusk (CT12). At night, these repressive complexes inhibit CLOCK/BMAL1-driven transcription in many CCG promoters, including their own, and are then progressively degraded. This relaxes the transcriptional repression before dawn, licensing the cycle to begin anew (Reppert and Weaver 2002). As more clock gene transcription factors and their co-complexes have been identified, sophisticated models of cycling transcriptional activation/repression with concomitant chromatin remodelling/histone modification have been developed and account for many experimental observations (Ukai and Ueda 2010). Indeed, the circadian

system has been a successful means of giving new life to well-established knowledge of transcriptional mechanisms, repackaging everyday factors into a clock context. Significantly, some clock genes, e.g. *Period1/2*, are immediate-early transcription factors, whose promoters also contain functional cAMP/Ca^{2+} response elements (CREs). In the SCN, in vivo and in vitro, appropriate activation of cAMP/Ca^{2+} signalling by extracellular (EC) stimuli induces *Period* gene expression and thereby facilitates clock resetting at night (entrainment) (Obrietan et al. 1999; Tischkau et al. 2003). These ideas and findings are reviewed elsewhere within this book (Buhr and Takahashi 2013; Sahar and Sassone-Corsi 2013), but in summary, the overarching hypothesis that has emerged to account for cellular timekeeping over the last two decades is one that posits cycling 'clock gene' transcription at its mechanistic core, with ancillary roles for post-translational mechanisms.

3.4 The Plot Thickens

Several recent findings have challenged whether cycling transcription is sufficient or even necessary to account for cellular timekeeping:

3.4.1 Transcriptional/Translational Feedback Loops Are Common

Transcriptional/translational feedback is a very common motif in cell biology (Kholodenko 2010) and could be described as the way a cell achieves proteostasis, i.e. a sufficient complement of protein activity to meet its requirements. In a signalling context, for example, a recurrent pattern is the rapid degradation of unstable inhibitors, a requirement for signal transmission, followed by their transcriptional up-regulation; this facilitates signal termination and a return to baseline (Legewie et al. 2008). For such oscillatory gene expression feedback loops, the time course is generally much shorter than 24 h, e.g. ERK signalling (2–3 h) (Kholodenko 2010) and NF-κB pathway (3–4 h) (Nelson et al. 2004), whilst the developmental segmentation clock has a period of 2–6 h (Jiang et al. 2000), reflecting a summation of the individual steps of gene expression (transcriptional activation/chromatin remodelling ⇒ elongation/splicing/5′-capping ⇒ termination/polyadenylation/nuclear export ⇒ translation ⇒ transport/translocation). Therefore, without positing a major contribution from post-translational processes, it is unclear why any transcriptional/translational cycle followed by cellular 'clock proteins' should take 24 h—it could all be done much more quickly.

3.4.2 Mismatches Between Transcriptome, Proteome and Protein Activity

With a couple of important exceptions, proteins mediate every cellular process of consequence. An uncontested biological principle is that protein sequences encoded

by DNA are transmitted through messenger RNA intermediates, leading to the common inference that cellular mRNA transcript levels correlate with the levels of protein that they encode. Recently, however, understanding of post-transcriptional regulation has advanced to the point that this cannot be assumed to be the case. Indeed, there are now numerous clock-relevant examples whereby protein activity is regulated post-transcriptionally, e.g. via interfering microRNA-mediated mRNA silencing (Cheng et al. 2007), alternative splicing (McGlincy et al. 2012), transcript-specific translational rate (Kim et al. 2007, 2010), global translational rate (Cao et al. 2011) or post-translationally through phosphorylation-directed, ubiquitin-mediated, proteasomal degradation (Eide et al. 2005; Reischl et al. 2007).

From a global cellular perspective, the evidence supporting a pre-eminent role for post-transcriptional regulation is compelling. Using microarray-based techniques numerous groups have reported that ~10 % of total mRNA transcripts across a range of tissues are circadian regulated. More recently, the cytosolic soluble proteome of murine liver and SCN was investigated across circadian time and showed that 10–20 % of proteins vary significantly over the circadian cycle. Strikingly though, no obligatory correlation was observed between a gene cycling at the mRNA vs. protein level (Deery et al. 2009; Reddy et al. 2006), i.e. rhythmic transcripts can encode proteins whose level is constant, rhythmic protein levels can be observed from transcripts of constant level and so on (Robles and Mann 2013).

The liver study also identified a number of proteins that were subject to rhythmic post-translational modification, e.g. peroxiredoxin (PRX) 6 exhibited a modification rhythm in anti-phase to protein and transcript levels. Such observations are critical since many protein activities are ultimately regulated by a cascade of covalent modification, and therefore, for genes implicated in clock mechanism, rhythmic transcript levels cannot be assumed to be of functional relevance since it is ultimately the spatio-temporal dynamics of protein activity that mediate biological responses.

3.4.3 Stochastic Effects/Gene Dosage

At the level of a single cell, transcription from a given locus is inherently noisy due to a combination of there only being 2 (or 1 on the X chromosome) copies/cell, the poor efficiency of successful transcriptional initiation and the frequency of RNA polymerase stalling (Blake et al. 2003; Wu and Snyder 2008). Such burst kinetics were recently shown directly for transcription from the *Bmal1* promoter (Suter et al. 2011). These stochastic effects might be expected to lead to highly variable cycle-to-cycle variation in period length rather than the ~10 % observed in isolated cells, in vitro. Moreover, dividing cells in G2 and ~40 % hepatocytes are polyploid ($\geq 4N$ chromosomes) (Gentric et al. 2012). Clearly, this would be expected to lead to gene dosage effects upon circadian period if the timing mechanism was mostly reliant on the timing of transcription. This has not, however, been observed calling into question a direct dependence of periodicity upon gene expression.

3.4.4 Lessons from Gene Over-Expression and Knockout

Over-expression or knockout of most so-called clock genes has negligible effects on the behavioural period in mice and cultured SCN (longer/shorter by <10 %) (Hastings et al. 2008), leading to the common interpretation that there is substantial redundancy between them (Welsh et al. 2010). In some instances (*Bmal1*$^{-/-}$, *Per2* over-expression, *Cry1*$^{-/-}$/*2*$^{-/-}$), mice are behaviourally arrhythmic (Bunger et al. 2000; Chen et al. 2009; Vitaterna et al. 1999), but this does not demonstrate that cellular rhythms have also been abolished. Indeed, where studied (*Bmal1*$^{-/-}$, *Cryptochrome1*$^{-/-}$/*2*$^{-/-}$), rhythms of circadian bioluminescence have been observed to persist in organotypic PER2::LUC SCN slices from these animals (Ko et al. 2010; Maywood et al. 2011). This robustness to genetic lesion implies that, at least in the SCN, the circadian circuitry is competent to maintain rhythmic PER2::LUC expression via other promoter elements and/or post-transcriptional regulation, even when rhythmic E-box activation is absent. Clearly, however, some basal activity of certain general transcription factors appears to be necessary for 'normal' cellular rhythms, but whether or not their rhythmic abundance is a prerequisite for timekeeping remains to be seen. These mammalian data have parallels with experiments performed in the fungus, *Neurospora crassa*, where the absolute necessity of identified 'clock genes' for cellular timekeeping was previously called into question (Lakin-Thomas 2006; Merrow et al. 1999; Granshaw et al. 2003).

3.4.5 Other Circadian Mutants: A Focus on Enzymes

It is notable that the circadian mutant mice with strongest period phenotypes carry dominant, apparently anti-morphic, mutations in genes that encode enzymes with roles in post-translational modification. For example, the *Clock*$^{-/-}$ mouse has a subtly shorter circadian period than wild type, whereas a mutation resulting in truncation of exon 19 that abolishes CLOCK's acetyltransferase activity results in a much longer period (~28 h in homozygotes) both in behavioural activity and cultured SCN (Debruyne et al. 2006; Vitaterna et al. 1994). Similarly, mice with homozygous deletions of casein kinase 1ε (CK1ε) have slightly longer circadian period (~0.5 h), whereas mice homozygous for the Tau (R182C) mutation exhibit a very short circadian period (20–21 h) (Meng et al. 2008). We infer from this that timekeeping competence is more susceptible to genetic perturbation of enzyme activity than direct disruption of identified transcriptional components.

3.4.6 Circadian Reporters: What Do They Report and Is It Enough?

Bioluminescent reporters for 'core' clock gene activity have been indispensable tools for delineating the complex, semi-redundant circuitry that facilitates temporal

coordination of physiology. In cases where genetic/pharmacological manipulation has resulted in the apparent loss of bioluminescence rhythms (arrhythmicity), however, absence of evidence is not evidence of absence. Indeed, one must consider whether the reporter, or the transcriptional circuit, and not the cellular clock per se has been affected. For example, the discrepancy in findings (arrhythmic vs. no effect) between different groups that have over-expressed the transcriptional repressor CRY1 in mammalian cell lines seems to be largely attributable to the length and nature of the promoter sequence that was used (Chen et al. 2009; Fan et al. 2007; Ueda et al. 2005). The emergent consensus is that cycling levels of most 'clock proteins' (e.g. CRY, BMAL1) are not required for timekeeping, but cycling PER is essential (Lee et al. 2011). This interpretation would be more compelling, however, were it supported by data using a clock reporter outside the genetic circuitry within which PER normally operates. In this context, the development of real-time post-translational reporters, not dependent on nascent gene expression, is highly desirable.

3.4.7 Clocks Continue Despite the Inhibition of Transcription

In all recent cellular clock models, the rate and timing of transcription constitute a key state variable. It was surprising, therefore, to learn that cellular rhythms in NIH3T3 fibroblasts are extremely robust to global inhibition of cellular mRNA production (using RNA polymerase II inhibitors, α-amanitin and actinomycin D). In this elegant study, >70 % of mRNA production was abolished during 3 days following drug treatment, and yet circadian period was shortened by <10 %; furthermore, the shortening was attenuated at lower temperatures, suggesting a hitherto unsuspected aspect of temperature compensation (Dibner et al. 2009). Similarly, using the PER2::LUC translational reporter, we observed that organotypic SCN slices exhibited at least one additional peak of bioluminescence during chronic treatment with α-amanitin oleate. At these concentrations (10–100 nM), we observed >70 % reduction in total ^{3}H-uridine incorporation in culture (Fig. 2a–c).

The above data are similar to recent findings in the marine alga *Ostreococcus tauri* (O'Neill et al. 2011). When cultures expressing either a transcriptional or translational clock gene::luciferase fusion were incubated with saturating concentrations of cordycepin (an inhibitor of total RNA synthesis), an additional cycle of correctly phased gene expression was observed only in the translational reporter cell line: not the transcriptional reporter (O'Neill et al. 2011). Taken together, these data might suggest that, provided the cell has sufficient mRNA in existence, non-transcriptional mechanisms are competent to sustain an additional cycle of clock-regulated protein synthesis. Indeed, in *Ostreococcus*, all transcriptional contributions to timekeeping seem to be restricted to subjective morning (CT0–8), since the reversible application of cordycepin to cultures outside this window has no effect on circadian phase following drug wash-off.

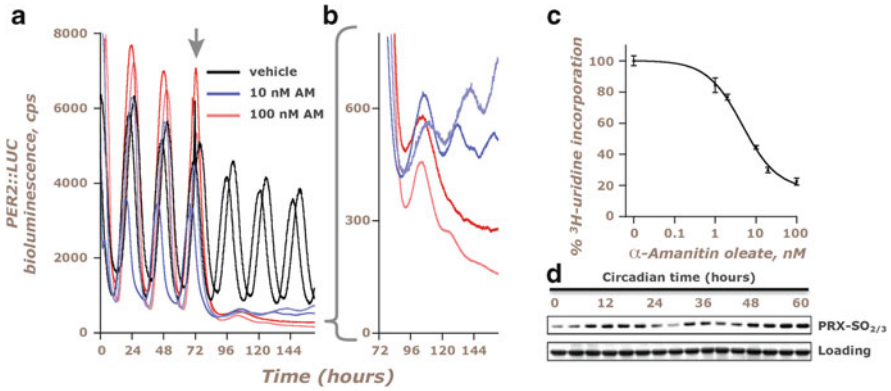

Fig. 2 Circadian rhythms persist in culture when transcription is inhibited or absent. (**a**, **b**) Representative and expanded plot showing rhythms in PER2::LUC bioluminescence persist in organotypic SCN slices during chronic treatment with α-amanitin oleate (AM, $n = 4$); *arrow* denotes start of drug treatment; (**c**) dose response for effect of AM upon nascent RNA synthesis using ^{3}H-uridine incorporation; (**d**) representative time course showing rhythms of PRX over-oxidation persist in isolated human erythrocytes (from O'Neill and Reddy 2011)

3.4.8 Clocks Continue Despite the Inhibition of Translation

In at least three unrelated experimental organisms, chemical 'wedge' experiments have been performed using the ubiquitous ribosomal inhibitor cycloheximide. The null hypothesis in such time courses posits that translation has no contribution to timekeeping at any point during the circadian cycle; any systematic deviations from the null hypothesis imply that it does. To perform these laborious experiments, translation is inhibited for increasing durations, beginning at different phases throughout the circadian cycle. In all three experimental models (mouse bioluminescent SCN slices, *Bulla gouldiana* ocular electrophysiology, *Ostreococcus* culture bioluminescence), approximately two-thirds of the circadian cycle ($\geq$16 h) was insensitive to translational inhibition, again implying that the majority of timekeeping function is not reliant on nascent gene expression (O'Neill et al. 2011; Khalsa et al. 1996; Yamaguchi et al. 2003).

3.4.9 Post-Translational Oscillations in Non-mammalian Systems

Extending these observations, there now exist several paradigms for cellular timekeeping in the complete absence of nascent gene expression. In *O. tauri*, it was recently shown that PRX post-translational rhythms persist for several cycles both in constant darkness (when transcription completely shuts down) and also in the presence of inhibitors of gene expression (O'Neill et al. 2011). This follows on from previous work in another alga *Acetabularia mediterranea*, where circadian rhythms of chloroplast movement were observed to persist when the nucleus of the

cell was removed (Woolum 1991). The landmark observations, however, were performed in the cyanobacteria *Synechococcus elongatus*, a prokaryote. Here it was shown that the ~24-h rhythm of KaiA/B/C protein phosphorylation and complex formation that occurs in living cells and normally interacts reciprocally with genome-wide transcriptional regulation could be reconstituted in vitro using just the three recombinant proteins (KaiA, B & C) with ATP (Nakajima et al. 2005). Bacterial expression systems tend to work on a 1 protein $\Rightarrow$ 1 function principle, whilst mammalian proteins tend to encode multiple domains with multiple, context-dependent cellular functions. We therefore think it unlikely that a directly equivalent experiment can be performed for mammalian timekeeping. It does raise the possibility, however, that the smallest functional circadian timekeeping unit may not include the nucleus.

3.4.10 Circadian Rhythms in Human Erythrocytes

Recently, the absolute requirement for nascent gene expression in mammalian cells was investigated in vitro. The ultimately cytotoxic effects of chronic inhibition of gene expression often confound pharmacological approaches to this problem. To circumvent this, preparations of human red blood cells (which are naturally anucleate) were employed. The rhythmic post-translational PRX modification, first observed in mouse liver, was used as a rhythmic marker. Briefly, the peroxiredoxin family constitutes a major part of the cellular defence against reactive oxygen species (ROS), specifically H_2O_2, which are an unavoidable by-product of aerobic metabolism. Erythrocytes express PRX at high levels (~1 % total protein), presumably due to the high ROS generation resulting from haemoglobin auto-oxidation. 2-Cys PRXs exist primarily as dimers that catalyse their own oxidation by H_2O_2 at conserved peroxidatic cysteine residues. The resultant sulphenic acid (Cys_P-SOH) may be reduced by a resolving cysteine on the opposing monomer (Cys_P-S-S-Cys_R) and ultimately reduced to the free thiol (SH) by the thioredoxin system. The kinetics of the resolving cysteine attack is quite slow, however, and in the presence of additional H_2O_2, over-oxidation to the sulphenic (Cys_P-SO_2H) or even sulphonic (Cys_P-SO_3H) form occurs (reversible through sulphiredoxin-catalysed, ATP-dependent mechanisms). By performing anti-2-Cys PRX-$SO_{2/3}$ immunoblots upon time courses of erythrocytes, isolated in a minimal glucose/salt buffer under constant conditions, circadian rhythms of PRX oxidation were observed (Fig. 2d). These rhythms were temperature compensated, entrainable by temperature cycles and robust to inhibitors of gene expression. In addition, the concentrations of several cellular metabolites ([ATP], [NADH], [NADPH]) appeared to be rhythmically modulated, as did an indirect fluorescence assay for haemoglobin multimeric state (O'Neill and Reddy 2011).

These data might suggest an underlying rhythmic capacity exists in the cytoplasm, not directly reliant on nascent gene expression, similar to the proposed 'cytoscillator' that we hypothesised previously (Hastings et al. 2008). At present, it is unclear whether the metabolic rhythms observable in isolated

erythrocytes are of direct physiological relevance, since other previously reported metabolic rhythms in cultured fibroblasts and mouse tissues, e.g. NAD^+ concentration, were attributed a transcriptional basis (Ramsey et al. 2009). It is interesting to note, however, that whilst cycles of PRX oxidation could be observed in transcriptionally arrhythmic mouse embryonic fibroblasts from CRY1/2 null mice, they were clearly perturbed compared to the more robust oscillation observable in the wild-type control. Although more work is needed, the implication is that in nucleated cells some post-translational metabolic rhythm interacts with (and probably reciprocally regulates) the defined transcriptional elements relevant to timekeeping.

3.4.11 High-Throughput Screening for Clock Regulators

A recent unbiased genome-wide, RNAi screen identified a number of genes whose downregulation significantly affected the period or amplitude of cellular rhythms (using two different bioluminescent reporters). Whilst RNAi-based approaches are frequently problematic due to off-target effects, it is telling that a significant proportion of 'hits' were identified as components of well-characterised metabolic and signalling pathways. Indeed, of the 12 strongest period phenotypes that were investigated in detail, knockdown of POLR3F and ACSF3 had no apparent effect on 'clock gene' expression, even though circadian period was increased or shortened, respectively (Zhang et al. 2009).

Several groups have also employed drug discovery approaches to identify compounds that affect timekeeping in cell culture. Although larger (often proprietary) library screens are still in progress, several data sets have been published (Chen et al. 2012; Hirota et al. 2008, 2011; Isojima et al. 2009) with roughly 1 % of compounds being observed to significantly affect circadian period. Several of these compounds confirmed the contribution of post-translational mechanisms already implicated in cellular rhythms (e.g. CK1δ/ε, GSK3β, adenylyl cyclase). In addition, novel regulatory mechanisms have been identified (e.g. CK1α; Hirota et al. 2011), as well as a number of inhibitors, agonists and antagonists that are reported to target proteins with no established role in cellular timekeeping (Isojima et al. 2009). Many of this latter group comprise membrane and intracellular signalling proteins. The implication of these 'discovery science' approaches is that significant numbers of cellular systems that contribute to the fidelity of timekeeping have not yet been integrated into any coherent model of cellular rhythms.

3.4.12 Conservation of Post-Translational Mechanisms Across Taxa

Across the eukaryotes, transcription factors implicated in timekeeping mechanisms are poorly conserved between phyla (Hastings et al. 2008; O'Neill et al. 2011). In contrast, a number of ubiquitous post-translational mechanisms are apparently

utterly conserved in their timekeeping roles, e.g. CK1, CK2, GSK3β, PP1/2A and proteasomal degradation (Hastings et al. 2008). Inhibitors of these enzymes have the same effects on cellular rhythms in the alga, *O. tauri*, as they do in mammalian cells (O'Neill et al. 2011), despite their divergence ~1.5 billion years ago. Whether this remarkable degree of conservation reflects a general requirement for certain housekeeping mechanisms, e.g. for targeted protein degradation, or conversely that these enzymes constitute part of a conserved post-translational timing mechanism that targets more recently occurring transcription factors is unclear. The paradigm of the conserved cell cycle role of eukaryotic cyclin-dependent kinases, and not their transcriptional targets, argues for the latter (Hastings et al. 2008). However, the striking similarity between the post-translational processing of clock proteins, e.g. PER2, with components of the wnt signalling pathway, e.g. β-catenin (Del Valle-Perez et al. 2011), argues for the former. Although there is some functional redundancy within each enzyme family, there being multiple isoforms, the activity of each is ultimately essential for cellular viability due to their participation in myriad cellular processes (see below). It is no surprise, therefore, that they are constitutively expressed. Interestingly though, some are reported to be rhythmically active, e.g. GSK3β due to their own circadian pattern post-translational modification (Iitaka et al. 2005).

As a marker for circadian timekeeping, intriguingly, the PRX oxidation rhythm appears to be particularly highly conserved, being observable in representative organisms from across the domains of life (Bacteria, Archaea, Eukaryota), unlike any TTFL component. Whilst PRX itself does not appear to play a critical timekeeping role, the redox rhythm it reports persists (albeit perturbed) in organisms that are deficient in 'core' TTFL components. We think it plausible that this remarkable conservation reflects either some underlying and ancient metabolic oscillation, which remains deeply embedded in the cellular machinery, or else an evolutionary convergence upon rhythmic redox regulation to facilitate temporal segregation of mutually antagonistic metabolic processes (Edgar et al. 2012).

4 Signalling Pathways and Metabolism

Whilst it is evident that contributions from transcriptional cycles to timekeeping are necessary for coordinated temporal organisation of normal physiology and behaviour and that transcription per se is ultimately required for life so that proteins/RNA can be synthesised, based on the points above, it is reasonable to posit that transcriptional cycles are not the mechanistic basis whereby circadian cycles take 24 h to complete. We will thus consider what other cellular processes might be relevant. The majority of implicated mechanisms, which are not classical transcription factors, are largely involved with signalling and/or metabolism; a few are discussed below, although many more components of well-known signalling pathways, e.g. mTOR and insulin/PI3K, are also increasingly being shown to play a role.

4.1 Second Messenger Pathways

Intracellular second messenger pathways are generally perceived to mediate rapid transmission of extracellular message to effector targets and thereby elicit the appropriate biological responses, e.g. change in ion channel activity, endo-/exocytosis, metabolic flux, transcriptional regulation, etc. Strikingly, however, circadian modulation of ubiquitous signalling systems, e.g. Ca^{2+}, cAMP, cGMP and nitric oxide, has been observed in a range of contexts (Hastings et al. 2008; Golombek et al. 2004), and their pharmacological manipulation has been demonstrated to affect timekeeping function. For technical reasons, it has not yet been possible to ascertain whether these reflect global changes in basal concentration as opposed to some clock-relevant subcellular spatio-temporal pattern of transients. Circadian crosstalk between these pathways, common in other signalling contexts, has not yet been investigated to any degree. Whilst previously considered an output from the 'core' oscillator and/or a means of entrainment, recent findings suggest that second messenger signalling makes direct mechanistic contributions to timekeeping itself (O'Neill et al. 2008). Since the majority of these experiments were performed in SCN organotypic slices, they will be discussed in the final section.

4.2 Phosphorylation and Other Post-Translational Modifications

From acetylation to sumoylation, from glycosylation to cysteine oxidation, all the major classes of post-translational protein modification have been implicated as regulating and/or being regulated by circadian timekeeping (Doi et al. 2006; Durgan et al. 2012; Gupta and Ragsdale 2011; Lee et al. 2008). This should be no surprise since if the cell is the clock, why should it restrict itself to any particular subset, of the biochemical tools at its disposal, with which to sculpt the spatio-temporal dynamics of whichever protein activities are relevant to timekeeping. Since protein phosphorylation is the best characterised of these, what follows is a brief description of the key components that have been identified to date.

4.2.1 CK1

CK1 family members are conserved, ubiquitously expressed Ser/Thr kinases that exist in an auto-phosphorylated inactive state, until dephosphorylated through activation of specific protein phosphatases. They have a wide range of cellular targets, both cytosolic and nuclear and regulate processes as diverse as membrane trafficking, DNA replication, wnt signalling and RNA metabolism. CK1 has a noted preference for phosphate-primed phosphorylation sites (Cheong and Virshup 2011).

Early mutagenesis screens in *Drosophila* revealed casein kinase1 (CK1) as a regulator of circadian period length, with different *doubletime* mutations leading

either to shortened or lengthened periods of rest-activity rhythms in flies (Kloss et al. 1998). In a remarkable parallel set of studies, the spontaneous *Tau* mutation of the Syrian hamster revealed the first circadian mutation in mammals, and this was later shown to involve an arginine to cysteine substitution within CK1ε, which caused a shortening of circadian period by 2 h for each copy of the mutated allele (Lowrey et al. 2000). In a further landmark discovery in humans, a group of familial sleep disorders characterised by early awakening were shown to segregate with mutations in human CK1δ or putative phosphorylation sites in human PER2. Subsequent genetic engineering of hamster and human mutations into mice has demonstrated how gain of function mutations of CK1δ and ε can enhance the rate of PER protein degradation, thereby accelerating the circadian cycle (Kloss et al. 1998). More recent genetic manipulations have shown that CK1δ and ε share overlapping roles in the pacemaker, and in the absence of both enzymes, the canonical transcriptional oscillator stops completely (Lee et al. 2011; Etchegaray et al. 2011; Meng et al. 2010). The development of selective inhibitors against CK1δ and ε has now made it possible, at least in animal studies and in tissue culture, to pharmacologically regulate circadian period, extending it to 30 h in a wild-type background and, by using a suitable dose, correcting to wild-type a shortened circadian period in CK1 mutants (Meng et al. 2010). Phosphorylation sites on PER2 regulated by wild-type and mutant CK1 are poorly characterised, but it is clear that, as with β-catenin, they license PER proteins for ubiquitinylation by the F-box protein β-TRCP and consequent proteasomal degradation (Reischl et al. 2007; Xu et al. 2009). Recently, pharmacological screening also implicated a hitherto unsuspected role for CK1α in the clock (Hirota et al. 2011).

4.2.2 CK2

CK2 is another ubiquitous and highly conserved protein Ser/Thr kinase that plays a central role in the control of a variety of pathways in cell proliferation, transformation, apoptosis and senescence (Montenarh 2010). It is composed of a catalytic dimeric α-subunit and a regulatory dimeric β-subunit. This complex is strongly implicated in regulating circadian rhythms in *Arabidopsis thaliana* (plant), *Neurospora crassa* (fungus) and *Drosophila melanogaster* (insect) and was recently identified in a large-scale functional RNAi screen to bind, phosphorylate and destabilise PER proteins in mammalian cells, probably acting synergistically with CK1 (Maier et al. 2009). Pharmacological inhibition of CK2 increases circadian period (Tsuchiya et al. 2009). Although many modes for activation have been reported, the upstream pathways for CK2 activation are unclear at present (Montenarh 2010).

4.2.3 GSK3

Glycogen synthase kinase-3 (GSK3) is a conserved and ubiquitously expressed multifunctional Ser/Thr kinase that was originally identified as a regulator of

glycogen metabolism. It plays a key role in numerous signalling pathways including regulation of the cell cycle, inflammation and cell proliferation (Xu et al. 2009). GSK3 is inactivated through phosphorylation by AKT/PKB in the insulin/PI3K signalling pathway, and spontaneous circadian cycling of GSK3 phosphorylation has been observed in cultured fibroblasts. This enzyme was originally implicated in timekeeping by over- and under-expression mutants in *Drosophila* that, respectively, decrease and increase circadian period (Martinek et al. 2001). There are two mammalian isoforms: α and β, with the GSK3$\beta^{-/-}$ mouse being embryonically lethal, due to this enzyme's essential role in development. In mammalian cells, pharmacological inhibition of GSK3 dose-dependently shortens circadian period (Hirota et al. 2008). GSK3β is reported to interact with clock proteins BMAL1, CLOCK, CRY2, PER2 and REV-ERBα, phosphorylate them and thereby regulate stability (Iitaka et al. 2005; Yin et al. 2006; Kurabayashi et al. 2010; Sahar et al. 2010; Spengler et al. 2009).

4.2.4 AMPK

5′-AMP-activated protein kinase is a ubiquitous and conserved, energy level sensor that acts as a metabolic switch that regulates several intracellular systems including the cellular uptake of glucose, the β-oxidation of fatty acids and mitochondrial biogenesis (Hardie 2011). It is a heterotrimer protein (α/β/γ, each having multiple isoforms). The α-subunit is catalytic (phosphorylating Ser/Thr), with the γ-subunit directly sensing AMP + ADP:ATP ratios (Xiao et al. 2011) but requiring additional phosphorylation by an upstream AMPK kinase for activity. Recently, AMPK was shown to phosphorylate and destabilise CRY1 and induce CK1-mediated degradation of PER2 in mammalian cells, with its activity and localisation being rhythmic in mouse liver (Lamia et al. 2009; Um et al. 2007).

4.2.5 Protein Phosphatases

Protein phosphorylation exists in dynamic equilibrium with phosphatase-mediated dephosphorylation. To date, the conserved and ubiquitously expressed protein phosphatase PP1 has been reported to regulate PER proteins (Lee et al. 2011; Schmutz et al. 2011), with PP5 being reported to modulate CK1ε activity in a CRY-dependent fashion (Partch et al. 2006). Based on observations in *Drosophila* and *Neurospora*, it seems likely that PP2A also plays some role in mammalian timekeeping (Sathyanarayanan et al. 2004; Yang et al. 2004).

4.3 Proteasomal Degradation

The functional contribution to cellular timekeeping made by the ubiquitous enzymes mentioned above has been interpreted in the context of net increases in

site-specific clock protein phosphorylation occurring over the circadian cycle, with some phosphorylation events promoting nuclear entry, but protein hyperphosphorylation licensing proteins for ubiquitin-mediated proteasomal degradation (Virshup et al. 2007). Because these means of regulating protein turnover are a well-established principle in cell biology and by no means unique to clocks (Xu et al. 2009; Westermarck 2010), it seems entirely plausible and is well supported by genetic and biochemical evidence.

4.3.1 F-Box and Leucine-Rich Repeat Protein 3: The *After-Hours* Mutation

By analogy with the observation that mutations affecting the phosphorylation of Per and Cry alter circadian period and then also changes in ubiquitinylation, the intermediary between (some) phosphorylations and proteasomal degradation should have a similar effect. This was demonstrated by two independent mutagenesis screens, which revealed long circadian periods in mice carrying point mutations in the C-terminal leucine-rich region of the F-box and leucine-rich repeat protein 3 (FBXL3), a component of SCF ubiquitinylation complexes (E3 ligases). In both mutations, Fbxl3 *Afterhour*s and Fbxl3 *Overtime*, circadian period of behavioural cycles and SCN bioluminescence rhythms is extended by *ca*. 1 h and 3 h in heterozygotes and homozygotes, respectively. This prolongation is ascribed to a reduced rate of CRY degradation, itself a consequence of a reduced affinity between mutant FBXL3 and its CRY substrates, which in turn slows down proteasomal targeting of CRY proteins (Godinho et al. 2007; Siepka et al. 2007). More recently, a second F-box protein, FBXL21, has also been implicated in the circadian clock as it also binds to, and directs for degradation, CRY proteins. It also compromises the negative-feedback actions of CRY on transactivation by CLOCK-BMAL1 complexes and is both highly enriched and rhythmically expressed in the SCN (Dardente et al. 2008). Interestingly high-throughput drug screening has recently revealed FBXL-mediated degradation of CRY as a novel target for pharmacological modulation of cellular timekeeping (Hirota et al. 2012).

4.4 Rhythmic Regulation of Protein Stability/Activity

In the context of the TTFL that has been proposed to account for cellular rhythms, current data suggest that a dynamic interplay between clock protein phosphorylation and dephosphorylation by these enzymes acts as an interval timer to regulate the kinetics of complex formation, protein degradation and nuclear entry, with certain specific serine/threonine residues on each clock protein substrate being implicated in tipping the balance between degradation and nuclear import (Virshup et al. 2007). An essentially identical model is proposed for wnt signalling, however, with the critical difference being that an upstream activating signal is required for stabilisation of β-catenin and its nuclear entry (Del Valle-Perez et al. 2011).

No such signal has been identified for clock protein regulation, although presumably something must act upstream to elicit the observed rhythms in kinase activity/clock protein stability.

Intuitively, any non-housekeeping protein must possess specific pathways for its synthesis and degradation to avoid erroneous expression and accumulation of oxidised/misfolded proteins. Being intrinsic to so many aspects of cellular signalling and metabolism, however, it is inconceivable to us that the kinases and phosphatases mentioned above could have specific roles purely in the regulation of clock gene transcription factors, since their hundreds of other cellular targets are not observed to be rhythmically regulated post-translationally. Given the known synergistic action of these and other clock-implicated kinases in the context of other protein substrates with multisite phosphorylation domains (Salazar and Hofer 2009), as also found in PER, it seems reasonable to us that most of the known transcription factor clock proteins act as cooperative, coincidence-detecting substrate effectors to amplify a low-amplitude modulation of enzyme activity within cellular signalling and metabolic systems, resulting in rhythmic clock protein activity, localisation and stability.

Again, by analogy with the cell cycle, a teleological justification for phase-specific activation and irreversible protein degradation is appealing since it would impart directionality to transcriptional elements of the circadian cycle. In the absence of external factors, which might stimulate additional clock protein synthesis, the slower kinetics of gene expression would impart robustness against perturbation to any purely post-translational oscillation we presume persists in isolated erythrocytes. In this context, transcriptional feedback repression of clock proteins would not be required for rhythmicity, but clearly would offer the advantage of positive signal amplification—since no signal can be transduced when the protein substrate is absent. This still leaves the question of what might act upstream to license post-translational rhythms in enzyme activity, which we posit are essential for circadian-regulated transcription factor activity/stability.

4.5 Metabolic Interactions

In a large number of experimental organisms, including mammalian cells and tissues, circadian rhythms in redox balance (e.g. NAD^+:NADH ratio), metabolite concentrations and coordinated metabolic processes (e.g. autophagy) have been reported (Minami et al. 2009; Merrow and Roenneberg 2001; Brody and Harris 1973; Powanda and Wannemacher 1970; Dallmann et al. 2012; Ma et al. 2011). For example, more than 20 years ago, reduced glutathione levels were reported to be rhythmic in isolated platelets, in vitro (Radha et al. 1985), and whilst platelets do still contain organelles, e.g. mitochondria and ribosomes, again the implication is that circadian rhythms can persist in the absence of (cycling) nuclear transcription, but not in the absence of metabolism, which is essential for cellular life.

It is interesting to note that several of the identified 'clock gene' transcription factors are haem-binding proteins and exhibit reciprocal regulation between rhythmic haem metabolism and the haem protein's redox/ligand status (Yin et al. 2007; Kaasik and Lee 2004; Dioum et al. 2002), e.g. haem binding and thus activity of the nuclear receptor REV-ERBβ are governed by a redox-sensitive cysteine (Gupta and Ragsdale 2011). Furthermore, the transcriptional activity of complexes containing the acetyltransferase CLOCK with BMAL1 and the antagonistic deacetylase SIRT1 is differentially regulated by the redox state of their NAD cofactors (Rutter et al. 2001; Nakahata et al. 2008; Asher et al. 2008). Thus, the activities of clock-relevant transcriptional factors would appear to be reliant upon metabolic state, whereas their localisation/stability would appear to be governed by intracellular signalling systems. Moreover, there are many established reciprocal pathways connecting redox balance and cellular metabolism with the activity of the various signalling mechanisms discussed above, e.g. (Cheong and Virshup 2011; Montenarh 2010; Hardie 2011; Vander Heiden et al. 2009; Sethi and Vidal-Puig 2011; Dickinson and Chang 2011; Metallo and Vander Heiden 2011). It is therefore entirely plausible to us that rhythms in the cytosol persist through cyclical, distributed crosstalk between multiple metabolic and signalling networks, with transcriptional clock components acting as coincidence-detecting substrate effectors that integrate the state of the network as a whole. In this context, irrelevant network perturbations would be ignored and appropriate extracellular cues responded to in a phase-dependent fashion. Rhythmic licensing of transcription, with its slower kinetics, would impart robustness to the 'cytoscillator' by rhythmic modulation of protein/transcript levels. Critically a rhythmic transcriptional contribution would not be required for oscillator competence, but the additional repression of clock protein activity upon its cognate gene and CCGs would facilitate signal amplification (Fig. 3).

5 SCN-Specific Timekeeping Mechanisms

5.1 SCN Physiology

SCN neurons exhibit several unusual features. Whilst circadian rhythms are ubiquitous in mammalian cells, the SCN exhibits much greater amplitude, robustness and accuracy resulting in, and from, increased interneuronal synchrony, i.e. amplitude and synchrony are mutually interdependent (Hastings et al. 2008; Abraham et al. 2010). For example, unlike other cultured tissues, the SCN appears to be resistant to entrainment by temperature cycles, unless its interneuronal communication is compromised (Buhr et al. 2010). What follows is a discussion of the genetic and pharmacological approaches that have been employed to delineate what makes the SCN so special, with particular emphasis on Ca^{2+} and cAMP signalling.

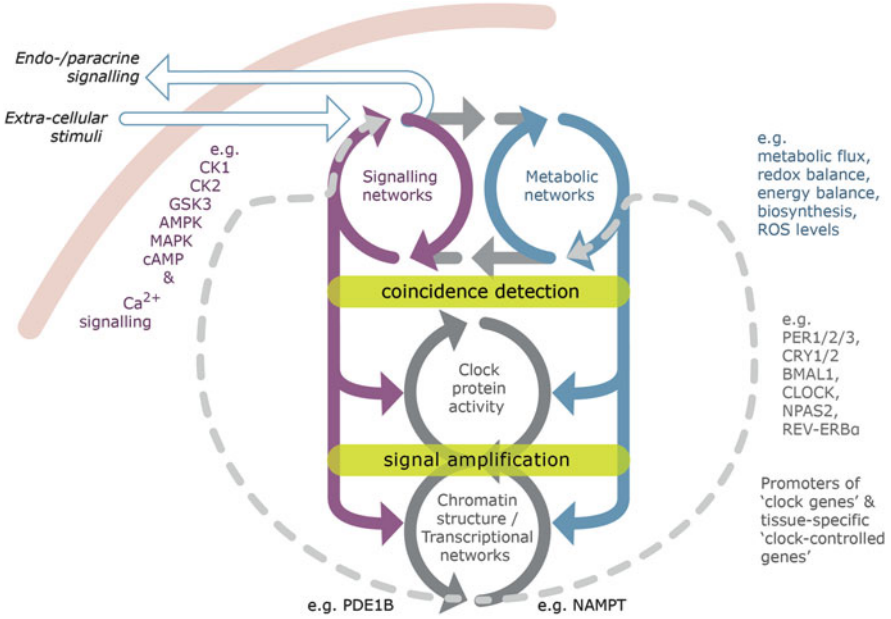

Fig. 3 A general model for cellular timekeeping. Circadian timekeeping is functionally distributed within the cell's metabolic and signalling networks and does not require nascent gene expression. In most (nucleated) cells, however, the integrated output from these networks is apparent in the circadian cycles of protein activity/stability/localisation observed, for example, in canonical clock protein transcription factors which act as 'coincidence detectors' for network state. These rhythmically modulate chromatin structure and facilitate coordinated temporal regulation of downstream transcriptional networks, including their own cognate clock gene circuitry, resulting in signal amplification. Rhythmic modulation of 'clock-controlled genes' facilitates coordinated temporal regulation of physiology and feeds forward into metabolic/signalling networks, modulating expression of some component mechanisms, e.g. rhythmic NAMPT expression facilitates rhythmic activity of the NAD^+ salvage pathway (Ramsey et al. 2009), and PDE1B degrades cAMP and affects rhythmic amplitude (Zhang et al. 2009). The circadian state of the signalling network modulates communication with local and distant targets, whilst selectively and temporally gating the capacity of relevant extracellular signals to affect circadian phase

SCN electrophysiology is overtly rhythmic, most neurons being more depolarised (~−50 mV) and spontaneously firing action potentials (APs) (ca. 10 Hz) during circadian day but hyperpolarised (~−60 mV) and silent (<1 Hz) at night (Pennartz et al. 2002; Colwell 2011). Blockade of neurotransmission (e.g. with tetrodotoxin, TTX) abolishes electrical rhythms and induces rapid damping of amplitude of circadian gene expression with progressive interneuronal desynchronisation (Yamaguchi et al. 2003). By implication, electrical excitability is required for coupling between individual cellular oscillators, making the whole greater than the sum of its parts. The axons of most SCN neurons project outwards to communicate with surrounding brain regions. Intra-SCN communication originates predominantly from exocytosis of dense-cored vesicles, principally from dendritic

sites (Castel et al. 1996). Vesicle release is mostly non-synaptic or parasynaptic, is Ca^{2+} dependent and may involve retrograde transmission facilitated by neural back-propagation (Gompf et al. 2006) and follow slower kinetics than for most excitable cells.

Metabotropic neuropeptide signalling appears to be essential to SCN timekeeping. Although functional electrical synapses exist, they are not required for timekeeping (Long et al. 2005). Similarly, no ionotropic neurotransmitter receptor has been demonstrated to be indispensable for SCN-intrinsic timekeeping, in vitro. For example, although intra-SCN synapses are mainly GABAergic, with most neurons synthesising/releasing GABA and expressing $GABA_A$ receptors, chronic inhibition of GABAergic signalling with bicuculline does not significantly affect timing (Gompf et al. 2006; Aton et al. 2006). GABA signalling does contribute to entrainment (Ehlen and Paul 2009) and modulate amplitude, however, possibly by restricting the extent of resting membrane depolarisation during the day whilst hyperpolarising it at night (Aton et al. 2006), perhaps acting in concert with a nightly K^+ channel efflux (Colwell 2011).

Several neuropeptides mediate SCN interneuronal communication, the foremost being vasoactive intestinal peptide (VIP) which binds the VIP/PACAP receptor (VPAC2), primarily signalling through adenylyl cyclase (AC) via Gsα (An et al. 2011). Auxiliary roles exist for gastrin-releasing peptide (GRP) and arginine vasopressin (AVP), both also signalling through their respective G-protein-coupled receptors to activate phospholipase C (PLC) (Gamble et al. 2007). VIP, GRP and AVP are expressed differentially in subpopulations throughout the SCN, although their receptors (particularly VPAC2) are more widely distributed (Welsh et al. 2010). Most likely, neuropeptide release occurs during the day in response to increased electrical activity facilitating vesicular exocytosis, thus allowing localised paracrine communication within the SCN network (Maywood et al. 2011).

Mice with homozygous deletion of genes encoding VIP or VPAC2 exhibit severely disrupted behavioural rhythms. The resting membrane potential in SCN neurons from these knockout mice (in vitro) is hyperpolarised, exhibiting reduced electrical activity, compared with wild type (Aton et al. 2005; Maywood et al. 2006). In SCN slices from homozygous VIP or VPAC2-null mice, molecular rhythms are profoundly affected. Most notably, the number of detectable bioluminescent neurons is substantially reduced relative to wild type, and rhythms in those neurons are stochastic, low amplitude and desynchronised from each other—similar to dissociated neurons or fibroblasts. Critically, several cycles of higher amplitude, synchronised rhythms can be rescued in VIP-null SCN by exogenous VIP (Aton et al. 2005). Similar observations have been made in VPAC2-null slices, treated with forskolin to directly activate AC. Rhythmic amplitude can similarly be rescued by GRP application, or by elevated intracellular Ca^{2+} (high $[K^+]_{EC}$) (Maywood et al. 2006). This implies that the timekeeping deficit in these animals can be ascribed to deficits in cAMP/Ca^{2+} signalling.

5.2 SCN Second Messenger Signalling

Second messenger signalling has long been viewed as an important means of cellular entrainment, e.g. in vitro, Glu elicits Ca^{2+}-mediated phase shifts in SCN, as does VIP acting via VPAC2/Gsα/AC/cAMP (Welsh et al. 2004; Brown and Piggins 2007; An et al. 2011). Moreover, circadian modulation of second messenger signalling has been reported as a rhythmic cellular output, e.g. in the SCN, both in vivo and in vitro, $[cAMP]_{cyto}$ varies ~fourfold, peaking shortly after projected dawn [~CT2 (O'Neill et al. 2008; Doi et al. 2011)]. Similarly, fluorescent probes reveal SCN $[Ca^{2+}]_{cyto}$ to be robustly rhythmic, again peaking shortly after projected dawn [~CT2 (Ikeda et al. 2003)]. It is significant that rhythms in both $[Ca^{2+}]$ and resting membrane potential are unaffected by TTX treatment (Pennartz et al. 2002). Intriguingly, the morning peaks and nightly nadirs in cytosolic cAMP and Ca^{2+} are coincident: with maximal activity occurring in advance of the peak in electrical activity (~CT6, midday) and so cannot be driven by it (Ikeda et al. 2003). Conversely, cAMP and Ca^{2+} are required for SCN electrophysiological excitability (Atkinson et al. 2011; Shibata et al. 1984). Signal transduction pathways have been established between elevated cAMP/Ca^{2+} and transcriptional activation via CREs, e.g. in the *Period1/2* promoter, so logically if second messenger signalling is both rhythmic output from, as well as input to, some hypothetical core clock mechanism, then dynamic cAMP/Ca^{2+} signalling becomes indistinguishable from that core mechanism (Hastings et al. 2008). Therefore, appropriate manipulation of cAMP and/or Ca^{2+} signalling should determine the key properties of cellular rhythms, i.e. amplitude, phase and period.

5.2.1 Effects upon Amplitude

Treatments that chronically elevate (forskolin + IBMX, pertussis toxin) or reduce (MDL12,330A) $[cAMP]_{cyto}$ induce dose-dependent damping of SCN rhythms and progressive interneuronal desynchronisation, in many respects phenocopying the VIP or VPAC2-null SCN. Normal rhythms return gradually following wash-off (O'Neill et al. 2008; Aton et al. 2006), revealing the self-organising properties of the SCN cells and circuit. Treatments that chronically elevate (ryanodine, high $[K^+]_{EC}$) or inhibit $[Ca^{2+}]_{IC}$ (Ca^{2+} chelators, low $[Ca^{2+}]_{EC}$, Ca^{2+} channel inhibitor cocktails) also dose-dependently reduce amplitude, with presumed desynchronisation (Maywood et al. 2006; Ikeda et al. 2003; Shibata et al. 1984; Lundkvist et al. 2005).

Chronically elevated/reduced cytosolic cAMP/Ca^{2+} levels also increase/decrease PER2::LUC baseline bioluminescence, respectively, stressing the critical contribution that CRE activation makes to clock gene regulation. Such manipulations reveal the dependence upon dynamic second messenger-mediated interneuronal coupling for the reciprocal interaction between amplitude and synchrony inherent to SCN timekeeping (Abraham et al. 2010).

5.2.2 Effects upon Phase

Following wash-off of forskolin + IBMX and subsequent decline of cAMP levels, SCN slices, regardless of prior phase adopt a common new phase, resetting to dusk (~CT12), when $[cAMP]_{cyto}$ normally approaches its nadir (coincident with the peak of PER2::LUC activity) (O'Neill et al. 2008). Glu-induced Ca^{2+}-mediated phase resetting of SCN has been reported extensively, being mimicked by Glu receptor agonists and blocked by antagonists (Kim et al. 2005). Ca^{2+} influx also resynchronises neurons in VPAC2-null SCN, and release of SCN slices from media with 0 mM KCl (low $[Ca^{2+}]_{cyto}$) resets internal phase to just after dawn (~CT3) when the cytosolic Ca^{2+} peak is normally observed (Maywood et al. 2006; Lundkvist et al. 2005). Thus, pharmacologically enforced cAMP/Ca^{2+} transitions override prior phase, forcing SCN phase to whenever such transitions would normally occur within the self-sustained circadian cycle.

5.2.3 Effects upon Period

Non-competitive (p-site) inhibitors of AC dose-dependently suppress SCN cAMP signalling (An et al. 2011), and reversibly, increase circadian period (to >31 h) in every tissue tested, in vitro, and are additive to manipulations that increase SCN period by other mechanisms (O'Neill et al. 2008). Increased mouse behavioural period, in vivo, was also observed when a p-site inhibitor (THFA) was delivered continuously and directly to the SCN via osmotic minipump (O'Neill et al. 2008). Whilst equivalent SCN experiments have yet to be performed for Ca^{2+}, the period of rat liver explants ex vivo was reversibly lengthened by pharmacological inhibition of endoplasmic reticulum (ER) Ca^{2+} store release and import, as well as membrane-permeable Ca^{2+} chelators (Baez-Ruiz and Diaz-Munoz 2011). Similar results may be expected from SCN slices.

5.3 SCN Timing and Second Messenger Crosstalk

Manipulation of SCN cAMP/Ca^{2+} signalling generally results in more marked SCN phenotypes than mutation/knockout of identified clock genes. Therefore, in addition to their other myriad biological roles (Hastings et al. 2008), dynamic cAMP/Ca^{2+} signalling critically contributes to SCN timekeeping, begging the question: which signalling proteins are involved, and how might crosstalk with circadian transcriptional elements be achieved?

Wild-type SCN slices exhibit daily cycles of elevated cAMP/Ca^{2+} culminating in rhythmic CRE activation at *Per* gene promoters, acting synergistically with rhythmic E-box activation, and it is presumed, thereby amplifying the oscillation. Within the CREB transcription factors, ATF4 has recently been implicated as one

such terminal effector (Koyanagi et al. 2011). The SCN-specific pathways that facilitate CRE activation are ill defined, but cAMP transduction certainly involves EPAC with an auxiliary role for PKA (O'Neill et al. 2008). For Ca^{2+}, the effectors CaMKII, MAPK and PKC have been similarly implicated (Welsh et al. 2010; Lee et al. 2010). Based on the extensive literature concerning synergistic effector regulation between the cAMP and Ca^2 signalling systems (Welsh et al. 2010), it is likely that the two normally operate in tandem to facilitate maximal CRE activation in the SCN.

It is unknown whether any AC isoforms preferentially participate in cellular rhythms, although the daily increase in SCN $Ca^{2+}{}_{cyto}$ signalling likely initiates primarily from intracellular stores. Whilst $Ins(1,4,5)P_3$ (IP_3) and ryanodine receptors (IP_3R, RyR) are certainly involved, the relative contributions made by different Ca^{2+}-mobilising messengers (IP_3, cADPR, NAADP) and Ca^{2+}-induced Ca^{2+} release remain poorly characterised. Although plasma membrane Ca^{2+} flux is required for SCN timekeeping, this is very likely an indirect consequence of the requirement for EC Ca^{2+} in vesicular exo-/endocytotosis (Schweizer and Ryan 2006), and for replenishing depleted intracellular stores during store-operated Ca^{2+} entry (SOCE) (Cohen and Fields 2006). Direct crosstalk between intracellular messenger systems, e.g. cAMP modulation of IP_3R activity (Schweizer and Ryan 2006), has not been investigated in the SCN.

The transcriptional clock circuitry within the SCN modulates cAMP/Ca^{2+} signalling through several means. For example, gene expression rhythms are observed for several SCN neuropeptides, neuropeptide receptors and AC/RyR isoforms, and functional contributions to timekeeping have been established, i.e. factors which enable rhythmic transcription (input) are themselves rhythmically expressed (output) (Welsh et al. 2010). Furthermore, daytime repression of Gi/o has been reported, through rhythmic expression of RGS16 (Doi et al. 2011). Most significantly, CRY1 was recently reported to directly inhibit Gsα activity in vitro and in mouse liver in vivo (Zhang et al. 2010); if this mechanism operates in the SCN, then it may well contribute to the decline of cAMP/Ca^{2+} late in the day, when CRY levels are increasing.

5.4 Daily Paracrine Positive-Feedback Coupling Within the SCN

It is known that elevated cytosolic cAMP and Ca^{2+} are competent to activate plasma membrane cation channels, e.g. cAMP regulates CNG channels to conduct mixed cation influx (Kaupp and Seifert 2002); SOCE induces Ca^{2+} and mixed cation influx via ORAI and TRPC channels, respectively (Cheng et al. 2011). Critically, persistent subthreshold cation channels exhibiting some similar properties to CNG/TRPC are observed in SCN slices (Kononenko et al. 2004), and to reiterate, compromised cAMP or Ca^{2+} signalling disrupts spontaneous electrical activity in SCN organotypic slices (Atkinson et al. 2011; Shibata et al. 1984), We propose it is therefore most

plausible that shortly after (projected) dawn, prior timekeeping mechanisms facilitate elevated cytosolic cAMP/Ca^{2+} signalling. This increases the 'open' probability of cAMP/Ca^{2+}-sensitive cation channels, thereby depolarising the resting membrane potential (~10 mV) and increasing AP firing probability. AP firing increases neuropeptide release, and neuropeptides act locally to stimulate further cAMP/Ca^{2+} signalling in neighbouring neurons (feedforward), which respond similarly. Clearly, this leads to positive feedback, sustained auto-amplification of cAMP/Ca^{2+} signalling within the SCN network, amplifying PER1/2 expression in the process, in the same phase as E-box activation. It is presumed that this sustained second messenger activity is relaxed by some combination of vesicular neuropeptide depletion, receptor desensitisation/internalisation and clock-driven modulation of the Gs/Gq/Gi/o transduction pathways, e.g. CRY1 and RGS16. Some redundancy must exist between Ca^{2+} and cAMP signalling for timekeeping within individual neurons, but we speculate that SCN-intrinsic encoding of projected dawn must rely upon the coincident detection of both second messenger systems being more active within the SCN network as a whole (see Fig. 4).

In a circadian context, second messenger signalling has not been studied extensively outside the SCN, although current data suggest that in fibroblast cultures also, a critical timekeeping role for dynamic changes in cAMP, Ca^{2+} and membrane potential exists (O'Neill et al. 2008; Noguchi et al. 2012). The above findings certainly provoke further questions, however. For example, is the timekeeping role of intracellular cAMP/Ca^{2+} rhythms ubiquitous in mammalian cells and simply with higher amplitude in the SCN, or specific to SCN timekeeping? What licenses the increased cAMP/Ca^{2+} signalling after dawn, and can inhibitors of gene expression block it? What specific spatio-temporal dynamic of SCN cAMP/Ca^{2+} signalling encodes timekeeping information: is it amplitude or frequency modulated (Berridge 1997); global, local or microdomain dependent? What is the contribution of other intracellular messengers, e.g. cGMP, and the crosstalk between them?

5.5 SCN Signalling in Context

Initiated by cell-intrinsic mechanisms, neuropeptide-mediated paracrine positive feedback between the extensively coupled neurons within the SCN facilitates a sustained increase in cAMP/Ca^{2+} signalling during the day. This accounts for the enhanced amplitude, robustness and precision of the SCN compared with other tissues that lack such coupling. Accordingly we presume SCN amplitude will be further reinforced, in vivo, when retinorecipient neurons receive photic inputs that evoke appropriate Ca^{2+} transients during the day. On the other hand, when those same transients occur at night, recipient neurons will phase-shift relative to non-recipient neurons, resulting in an increased phase distribution across the SCN. Due to SCN positive-feedback coupling, however, this transitory phase dispersal is integrated to become more coherent during the following day, resulting in a modest

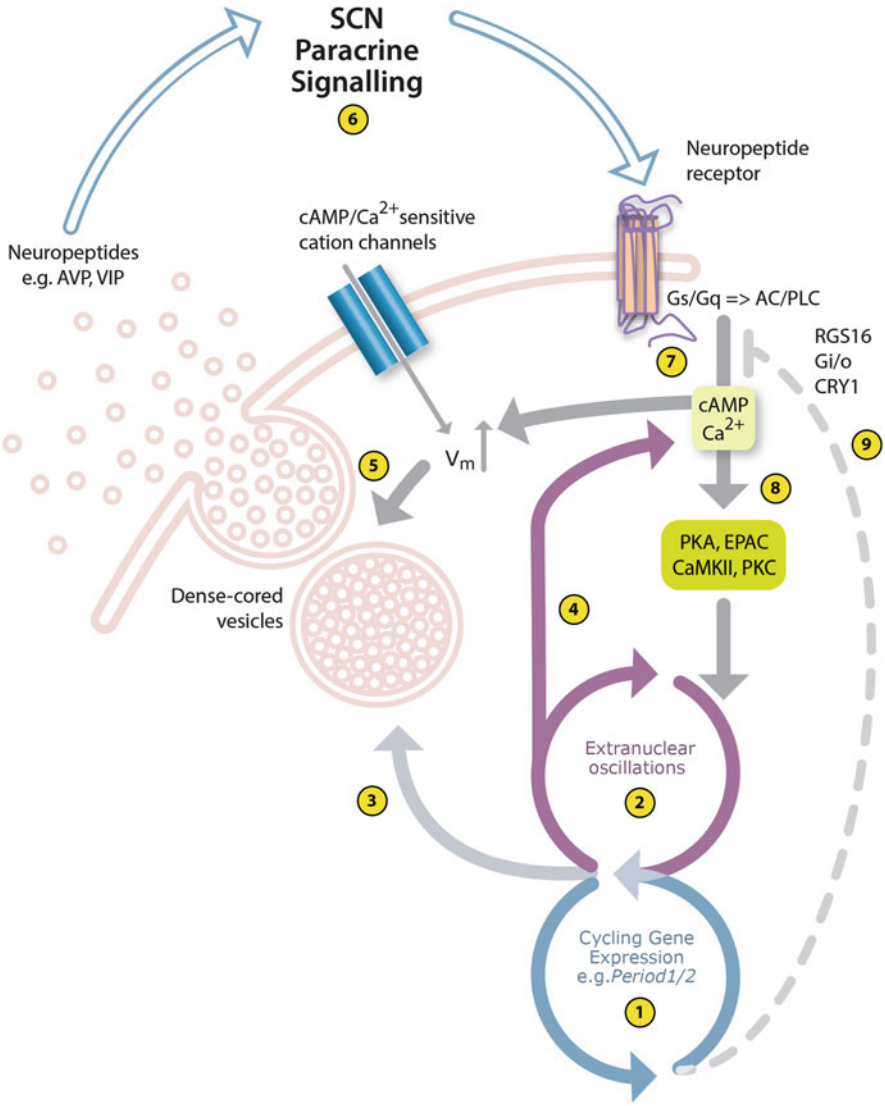

Fig. 4 Schematic of SCN paracrine positive-feedback coupling from a single neuron perspective. Cellular timekeeping normally results from reciprocal crosstalk between transcriptional/translational feedback loops (1) with extranuclear oscillations (2) in signalling and metabolism to facilitate rhythmic regulation of clock-controlled genes (3), e.g. AVP, and also increased cAMP/Ca^{2+} signalling around anticipated dawn (4). cAMP/Ca^{2+} depolarises resting membrane potential (V_m), thereby increasing electrical activity and neuropeptide release (5), further elevating cAMP/Ca^{2+} signalling within the network, eliciting further neuropeptide release (6). Neuropeptide receptor activation amplifies cAMP/Ca^{2+} signalling (7) and activates downstream effectors (8). Later in the day, cAMP/Ca^{2+} signalling decreases through some combination of neuropeptide depletion and/or receptor desensitisation/internalisation and/or change in gene expression of inhibitors of G-protein signalling (9)

phase shift by the network as a whole. Thus, whilst our understanding of intracellular timekeeping is still lacking in mechanistic detail, the reason why the SCN does it better is becoming increasingly clear.

6 General Conclusion

A large number of cellular components relevant to timekeeping ('clock genes/proteins') have been identified to date. Of late they have tended to be enzymatic or metabolic in nature, rather than transcriptional. As with most aspects of eukaryotic cell biology, their role is not specific to circadian timekeeping and is usually redundant within it. The era when research papers proclaimed 'gene X' or 'process Y' plays a role in circadian timekeeping is coming to an end, since the emergent theme seems to be one whereby the activity of some cellular/biochemical process which is rhythmically regulated can in turn feedback into regulating that rhythm and thereby becomes indistinguishable from the core mechanism. In a cycle, it is impossible to separate cause from effect, and yet if the cell itself is the oscillator and can utilise any of the numerous tools at its disposal to sustain rhythms, how do we progress towards a detailed mechanistic understanding? We envisage three approaches:

(1) Pragmatic—It does not matter how the cellular oscillator works. There is a wealth of knowledge concerning how circadian rhythms impact upon biology, and that understanding can be applied productively now.
(2) Systems biology—Circadian rhythms are an emergent property of mammalian cells and cannot be understood at a level simpler than the cell. In order to understand this timekeeping phenomenon, we must understand or have data about all relevant aspects of cell biology. Since the behaviour of a sufficiently complex system cannot be grasped intuitively, the model must be built in silico, being able to make nonintuitive predictions that can be tested and the model refined, iteratively.
(3) Reductionist—There is a core oscillatory mechanism from which all the other identified timekeeping systems are driven, but ultimately feedback into. If this core oscillator can be sufficiently reduced, it can be understood biochemically and a model built from the bottom up.

For the pharmacologist, the first option should be the most attractive. The tools required to test whether a new or existing drug affects cellular rhythms, or whether rhythms affect its pharmacokinetics/dynamics, already exist, and it is hoped that this and the other chapters in this volume will help catalyse such endeavours.

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The Clock in the Brain: Neurons, Glia, and Networks in Daily Rhythms

Emily Slat, G. Mark Freeman Jr., and Erik D. Herzog

Abstract The master coordinator of daily schedules in mammals, located in the ventral hypothalamus, is the suprachiasmatic nucleus (SCN). This relatively small population of neurons and glia generates circadian rhythms in physiology and behavior and synchronizes them to local time. Recent advances have begun to define the roles of specific cells and signals (e.g., peptides, amino acids, and purine derivatives) within this network that generate and synchronize daily rhythms. Here we focus on the best-studied signals between neurons and between glia in the mammalian circadian system with an emphasis on time-of-day pharmacology. Where possible, we highlight how commonly used drugs affect the circadian system.

Keywords SCN • VIP • GRP • AVP • Little SAAS • GABA • ATP

1 Neurons of the SCN

The nearly 20,000 neurons of the suprachiasmatic nucleus (SCN) have been identified as the alarm clock, or master circadian pacemaker, to the remaining 100,000,000,000 neurons in the human brain (Klein et al. 1991). Put succinctly, the SCN has been ascribed a single function—to synchronize the body's daily rhythms to local time. Although most of the evidence comes primarily from mice, rats, and hamsters, the SCN appears to be highly conserved in its anatomical and physiological organization. The SCN acts as a central timer in vivo and in vitro. In vivo, multiple brain regions exhibit circadian changes in electrical activity, with the SCN peaking during the day and the others at night (Inouye and Kawamura 1982;

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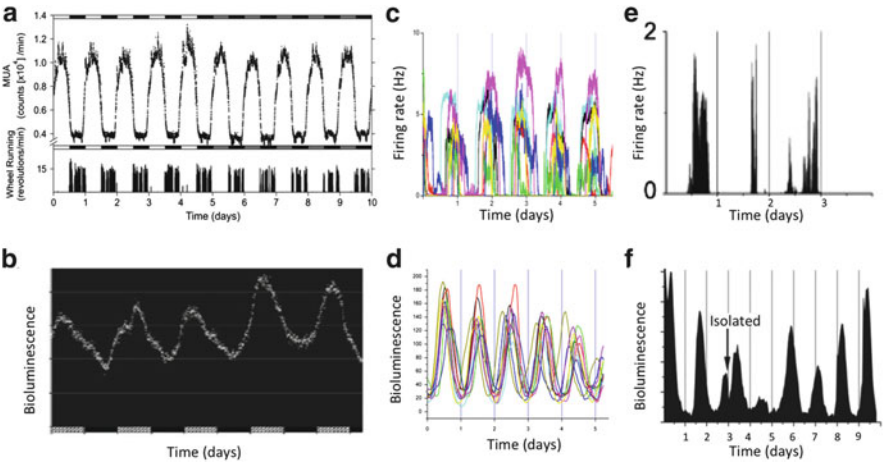

Fig. 1 Pharmacology of the circadian system must be considered in the context of daily changes in gene expression and membrane excitability. SCN neurons are capable circadian pacemakers in vivo, in vitro, and in isolation. (**a**) In vivo multiunit firing rhythms in the SCN. (**b**) In vivo Per1 transcription rhythms in the SCN. (**c**) In vitro firing rhythms recorded from 10 representative neurons with synchronized circadian periods. (**d**) In vitro Period1 rhythms from 10 representative cells with synchronized circadian periods. (**e**) Isolated SCN neuron shows rhythm in firing rate. (**f**) Isolated SCN neuron shows daily rhythms in Period2 protein expression

Yamazaki et al. 1998; Meijer et al. 1998) (Fig. 1). Ablation of the SCN abolishes many of these coordinated daily rhythms in the brain and behavior (Ralph et al. 1990; Moore and Eichler 1972; Stephan and Zucker 1972). Critically, SCN transplants restore behavioral circadian rhythms in SCN-lesioned animals with the period of the donor (Ralph et al. 1990; Sujino et al. 2003). When isolated in vitro, the SCN continues to express circadian rhythms in glucose metabolism, gene expression, neuropeptide secretion, and electrical activity similar to its rhythmicity in vivo (Green and Gillette 1982; Earnest and Sladek 1986; Shinohara et al. 1995; Herzog et al. 1997; Quintero et al. 2003; Yamazaki et al. 2000) (Fig. 1). Thus, the SCN acts as a pacemaker that generates and drives daily rhythms in the brain and body.

Individual SCN neurons are competent circadian pacemakers. Just as single cyanobacteria and isolated retinal neurons from a marine snail show circadian oscillations (Mihalcescu et al. 2004; Michel et al. 1993), SCN neurons have recently been shown to cycle on their own (Fig. 1) (Webb et al. 2009). This is consistent with the standard model in which intracellular molecular events regulate daily rhythms in transcription and translation (Welsh et al. 2010). SCN cells retain many of their circadian properties when isolated from their network such as a genetically determined period near 24 h that changes little over a wide range of temperatures (Herzog and Huckfeldt 2003). Importantly, when isolated either physically or pharmacologically from their neighbors, SCN cells lose their daily precision and become relatively unstable oscillators (Webb et al. 2009; Liu et al.

2007; Abraham et al. 2010). The SCN thus comprises a multi-oscillator system that depends on intercellular signaling to synchronize the component oscillatory cells to each other and to environmental cycles.

The population of heterogeneous SCN neurons has spatial organization. Anatomically, the SCN has been divided into a dorsal shell and ventral core (Moore et al. 2002; Antle et al. 2003, 2007; Morin 2007). The retinal inputs are most dense in the ventral SCN where light first induces immediate genes (e.g., cFOS and Period1; Hattar et al. 2002; Abrahamson and Moore 2001). The dorsal SCN has been noted for its circadian rhythms in gene expression and as a recipient of projections from the ventral SCN (Leak and Moore 2001). Indeed, there are lighting conditions that can force the rhythms in the dorsal and ventral SCN apart, supporting a model where the ventral SCN lacks intrinsic oscillations and conveys photic information to the intrinsically rhythmic neurons of the dorsal SCN (Karatsoreos et al. 2004; LeSauter et al. 1999; Shigeyoshi et al. 1997). However, there is also strong evidence that cells in both the top and bottom of the SCN are intrinsically circadian (de la Iglesia et al. 2004; Cambras et al. 2007; Yamaguchi et al. 2003; Shinohara et al. 1995; Albus et al. 2005; Webb et al. 2009). It is not yet clear whether some, most, or all SCN cells are functional circadian pacemakers.

1.1 Neuron–Neuron Signaling in the SCN

Although intercellular communication within the SCN has been the focus of significant experimental effort, little is known about how SCN cells synchronize to each other to coordinate behavior. Most neurons within cultured explants of the SCN express synchronized circadian rhythms (Herzog et al. 1997; Quintero et al. 2003; Yamaguchi et al. 2003; Nakamura et al. 2001), while neurons dispersed at low density tend to oscillate with different periods (Welsh et al. 1995; Liu et al. 1997b; Herzog et al. 1998; Honma et al. 1998b; Nakamura et al. 2002). Dispersed SCN cells, when transplanted into SCN-lesioned animals, restore circadian behaviors (Silver et al. 1990) and, when plated at higher densities in vitro, secrete vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) (Murakami et al. 1991; Honma et al. 1998a) in a coordinated circadian pattern. This indicates that SCN cells release and receive signals that allow them to synchronize to each other.

The list of candidate intercellular signals within the SCN is extensive and virtually unexplored. We must consider factors that could be secreted by neurons or glia through vesicular and non-vesicular release mechanisms. For example, a screen for genes expressed in the SCN that encode secreted and membrane-bound proteins identified more than 100 peptides, including growth factors, cytokines, chemotrophins, neuropeptide precursors, and transmembrane proteins that signal after cleavage (Kramer et al. 2001). A recent effort to sequester and sequence peptides secreted from SCN explants identified more than 100 peptides derived from 27 precursor proteins (Lee et al. 2010). These lists will include at least some of the synaptic and extrasynaptic releasates but will miss signals carried through gap

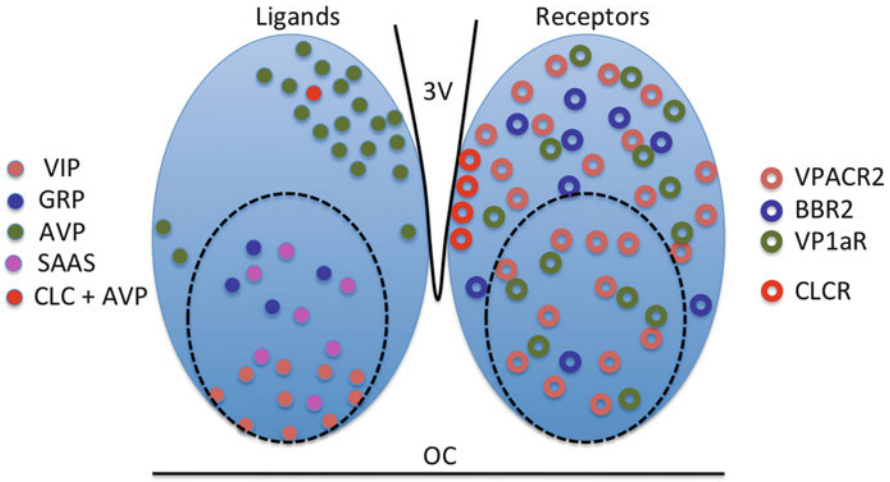

Fig. 2 Schematic localizing ligands and their cognate receptors in the SCN. For simplicity, the *left* SCN illustrates distributions of cells expressing identified ligands, and the *right* SCN shows somata expressing the relevant receptors. Based on neuropeptide expression, five distinct classes of cells account for approximately 50 % of the neurons in the SCN. These peptidergic cell classes are AVP, VIP, GRP, little SAAS, and CLC. Each *filled circle* represents the cell-body location of approximately 100 neurons. The broad distribution of the cognate receptors (*open circles*) in the SCN (largely based on mRNA expression) suggests extensive and convergent signaling from these distinct classes within the SCN. Here, CLCR-positive cells express the three genes believed to encode the heterotrimeric CLC receptor. The *dashed circles* delimit the area of densest retinal innervation often termed the SCN core. *3V* third ventricle, *OC* optic chiasm

and hemi-junctions. Here, we focus on the pharmacology of a short list of signals that have been most studied (Fig. 2). For each, we review the ligand and receptor, the time of day when most effective, the signaling cascade, and the potential role in SCN function.

1.1.1 VIP/VPAC2R

Produced by approximately 10–22 % of SCN neurons (Abrahamson and Moore 2001; Atkins et al. 2010; Moore et al. 2002), VIP is at the top of the hierarchy of influential signals in the SCN. Deletion of the *VIP* gene or the *Vipr2* gene for the VIP receptor, VPAC2R, results in the most severe circadian phenotype of any signaling molecule studied thus far: disrupted circadian behaviors and hormonal secretion, 8-h advance of the daily onset of activity in a light–dark cycle (i.e., phase angle of entrainment), and drastically reduced synchrony among circadian cells in the SCN (Maywood et al. 2011b). VIP neurons are primarily located in the ventral SCN where they receive dense innervation from the retina (Harmar et al. 2002; Hattar et al. 2002). VIP induces calcium influx (Irwin and Allen 2010) and changes in firing rate (Reed et al. 2001) and shifts the phase of the SCN through parallel

increases in adenylate cyclase and phospholipase C activities (An et al. 2011). Although *Vipr2* mRNA appears throughout the SCN (Usdin et al. 1994; Kalamatianos et al. 2004; Kallo et al. 2004), it is not yet clear if VIP acts directly on all or a subset of SCN cells.

VIP likely plays a role in synchronization between circadian cells and adjustments to the light–dark cycle. VIP is released in a circadian pattern from the cultured SCN (Shinohara et al. 1993, 1995), but there is conflicting evidence for circadian VIP release in vivo (Laemle et al. 1995; Francl et al. 2010). VIP release is stimulated also by light in vivo (Francl et al. 2010; Shinohara et al. 1998).

Importantly, the effects of VIP depend on the time of administration. During the subjective day and early subjective night, VIP dose-dependently delays circadian rhythms in the SCN with a maximal effect around subjective dusk (Reed et al. 2001; An et al. 2011). During the late subjective night and early morning, VIP modestly advances the SCN. When applied daily, VIP entrains the isolated SCN (An et al. 2011). These results are consistent with a model in which the 3,000 VIPergic neurons of the SCN synchronize their circadian rhythms to each other and coordinate circadian timing throughout the SCN.

To better understand whether VIP acts alone or in concert with other signals to synchronize SCN cells, Maywood and colleagues developed a novel coculture technique (Maywood et al. 2011b). They took advantage of the VIP-deficient SCN in which cells fail to synchronize their daily rhythms in gene expression. They found that a wild-type SCN could restore coordinated circadian cycling in a VIP-deficient SCN explant, confirming that VIP is both necessary and sufficient for sustained rhythms in the SCN and revealing that VIP can diffuse several millimeters to accomplish this task. However, when they discovered that wild-type SCN could slowly restore circadian rhythms to VPAC2R-deficient SCN, they concluded that other signals also must be capable of synchronizing SCN cells.

1.1.2 GRP/BBR2

Produced by approximately 4–10 % of SCN neurons (Abrahamson and Moore 2001; Antle et al. 2005; Atkins et al. 2010; Moore et al. 2002), gastrin-releasing peptide (GRP) in the SCN appears to have functions similar to and distinct from VIP. GRP-synthesizing neurons are found in the middle of the SCN and GRP receptor (BBR2). mRNA appears throughout the SCN, with more in the dorsal SCN (Aida et al. 2002; Karatsoreos et al. 2006). It is not yet clear if all or some SCN neurons respond directly to GRP.

Like VIP neurons, GRP neurons have been implicated in the SCN response to nighttime light exposure. Like VIP neurons, they receive retinal input and respond to nocturnal light with increased transcription of *cFOS* and the *Period* genes (Bryant et al. 2000; Karatsoreos et al. 2004). It is not yet clear if light induces the release of GRP. Like VIP, GRP signals through increases in cAMP (Gamble et al. 2007) and has been implicated also in synchronization between circadian SCN cells. GRP application in vivo and in vitro can shift SCN rhythms (Piggins et al.

1995; Antle et al. 2005; Kallingal and Mintz 2006; Gamble et al. 2007). Although blocking GRP receptors (BBR2) does not abolish SCN rhythms, it prevents SCN cocultures from restoring rhythms to VIP-deficient SCN (Maywood et al. 2011b). Finally, GRP application induces coordinated circadian rhythms in SCN deficient for VPAC2R (Brown et al. 2005; Maywood et al. 2006). Taken together, these results support the hypothesis that GRP is a weaker synchronizing agent than VIP but participates in entrainment of SCN circadian rhythms.

Anatomical data suggest an additional role for GRP neurons in communicating information from the dorsal to ventral SCN (Drouyer et al. 2010). In one model, synchrony among the circadian cells of the SCN requires this feedback onto retinorecipient SCN cells to gate their sensitivity to ambient light (Antle et al. 2007). Consistent with this hypothesis, mice lacking the GRP receptor show attenuated shifts to bright light (Aida et al. 2002). It will be exciting to see if animals deficient for GRP are slower to adjust to shifts in their light schedule and whether their dorsal and ventral SCN might fail to remain synchronized.

1.1.3 AVP/V1aR

Produced by approximately 20–37 % of SCN neurons (Abrahamson and Moore 2001; Moore et al. 2002), arginine vasopressin (AVP) was the first neuropeptide discovered in the SCN, although years after it was found in the magnocellular neurosecretory hypothalamic (supraoptic and paraventricular) nuclei where it is produced in even greater abundance (Swaab et al. 1975; Vandesande et al. 1974; Burlet and Marchetti 1975). AVP-synthesizing neurons are found in the dorsal-medial SCN and, in mouse, also in a small group of magnocellular neurons in the lateral SCN. In the SCN, AVP signals primarily through V1a receptors which appear to be broadly expressed (Li et al. 2009). Although most SCN neurons increase their firing in response to AVP, it is not yet clear if the response is direct or through network interactions (Ingram et al. 1996).

The regulation of circadian AVP release occurs at the levels of transcription (Jin et al. 1999), translation, and neuronal excitability. AVP levels in the cerebrospinal fluid vary with time of day, depending on the SCN, with a morning peak about five times higher than in the evening (Abrahamson and Moore 2001; Moore et al. 2002). This rhythm is intrinsic to the isolated SCN (Swaab et al. 1975; Vandesande et al. 1974; Burlet and Marchetti 1975) and regulated, at least in part, through circadian transcription (Li et al. 2009) and polyadenylation (and subsequent translation) of the transcript (Ingram et al. 1996). Interestingly, the rhythm in AVP transcription depends on neuronal firing, VIP, cAMP, and Ca^{2+} signaling (Reppert et al. 1981; Jansen et al. 2007; Sodersten et al. 1985; Tominaga et al. 1992). This provides a nice example of how intercellular signaling and intracellular transduction cascades in the SCN are critical for the daily rhythms from gene expression to neuropeptide secretion.

AVP has been primarily implicated in regulating the amplitude of circadian rhythms in the SCN and paraventricular nucleus of the hypothalamus (Tousson and

Meissl 2004) and in hormone and behavior rhythms (Gerkema et al. 1999; Jansen et al. 2007). In contrast to animals deficient for VIP, animals deficient for AVP or the V1a receptor display circadian rhythms with normal periodicity but with attenuated amplitudes (Li et al. 2009). AVP-deficient Brattleboro rats display low-amplitude daily rhythms in sleep-wake, body temperature, plasma melatonin, and SCN firing rates. Similarly, AVP levels and periodicity in the SCN of common voles correlate with the amplitude of their locomotor rhythmicity. Mice lacking V1aR show diminished rhythms in locomotion and in expression of at least one gene, *prokineticin 2*, in the SCN (Robinson et al. 1988). Because AVP is likely released during the day when firing rates are high in the SCN and excites most SCN neurons, it is possible that AVP regulates the gain of the SCN and drives rhythms in downstream targets (Rusnak et al. 2007).

Coculture experiments have recently suggested an additional role for AVP in the SCN. Much like GRP, blocking AVP receptors does not abolish SCN rhythms in vitro, but does prevent SCN cocultures from restoring rhythms to VIP-deficient SCN (Maywood et al. 2011a). It is possible that AVP normally amplifies SCN rhythms and, when VIP signaling has been compromised, AVP, alone or through GRP, can act as a weak synchronizing agent to coordinate the rhythms among the many circadian cells of the SCN.

1.1.4 Little SAAS

The recent reports on little SAAS exemplify novel approaches to discovering new molecules involved in circadian communication. Historically, SCN signals were identified when a good antibody existed. Little SAAS emerged from a relatively unbiased screen for secreted molecules in the SCN (Hatcher et al. 2008). In this approach, spontaneous and electrically evoked releasates were concentrated from explanted SCN and characterized by mass spectrometry. Further improvements with a 12 Tesla LTQ-FT mass spectrometer and ProSightPC 2.0 software led to the identification of 102 endogenous peptides released from the SCN including 33 novel peptides and 12 with posttranslational modifications including amidation, phosphorylation, pyroglutamylation, or acetylation. These methods allow for simultaneous identification of many signals from identified tissues or even areas within the SCN under a variety of stimulation conditions.

Because it was in relatively high abundance and putatively involved in prohormone processing, little SAAS rose to the top of the list of peptides to be further characterized by the labs of Martha Gillette and Jonathan Sweedler. Produced by approximately 16 % of SCN neurons (Maywood et al. 2011b), little SAAS signaling in the SCN appears to have functions similar to and distinct from VIP and GRP. Little SAAS-synthesizing neurons are found primarily in the middle of the SCN. Approximately 33 % of them do not express either GRP or VIP, but the 67 % remaining represent 80 % of GRP- and 10 % of VIP-positive neurons (Hatcher et al. 2008). This suggests that little SAAS may be co-released, at least under some conditions, with other neuropeptides.

Like VIP and GRP, little SAAS has been implicated in the SCN response to nighttime light exposure. Neurons positive for little SAAS receive retinal input and respond to nocturnal light with increased cFOS (Lee et al. 2010). It is not yet clear if light induces the release of little SAAS, but electrical stimulation of the optic nerve increases little SAAS release (Fricker et al. 2000). Remarkably, an antibody to little SAAS can block glutamate-induced delays of the in vitro SCN (Atkins et al. 2010). In addition, little SAAS application in vitro can shift SCN rhythms (Hatcher et al. 2008) independent of VIP or GRP signaling (Atkins et al. 2010). Taken together, these results support the hypothesis that little SAAS signals in parallel to or independent of GRP and VIP in response to light.

This may indicate a high level of redundant functions for different neuropeptides in photic entrainment. Alternatively, we may need more sophisticated assays to distinguish their roles with higher spatial and temporal resolution and under diverse conditions. This is exemplified in central pattern generators where circuit properties depend on which neuropeptides are released (Dickinson 2006; Wallén et al. 1989). It will be exciting to see, for example, if animals deficient for little SAAS (Atkins et al. 2010) fail to entrain to specific light cycles or initiate their daily activity at abnormal times.

1.1.5 GABA

Most, if not all, of the diverse peptidergic neurons of the SCN share one important function—they synthesize γ-aminobutyric acid (GABA) (Belenky et al. 2007). Both the ionotropic, $GABA_A$, and metabotropic, $GABA_B$, receptors are expressed widely in the SCN and on the terminals of projections to the SCN (Gao et al. 1995; Belenky et al. 2003, 2007; Francois-Bellan et al. 1989). Thus, GABA is postulated to act directly on all SCN neurons and on inputs to the SCN.

Although it is rare in the adult nervous system, there is good evidence that GABA can excite neurons of the SCN. GABA was first reported in 1997 as excitatory during the day and inhibitory at night, thus amplifying the daily rhythm in firing rate (Wagner et al. 1997). Unfortunately, the time-of-day effect has not been reproducible with different labs reporting excitation by GABA: during the night (Pennartz et al. 2002), during the night in only the dorsal SCN at all times but in a fraction of the dorsal and ventral SCN neurons (Choi et al. 2008; Irwin and Allen 2009), or never (Gribkoff et al. 2003; Liu and Reppert 2000; Aton et al. 2006). Some of this confusion may be explained by difficulties in defining responses as excitatory when they reflect post-inhibitory rebound. It is, however, reasonable to conclude that GABA likely excites a subset of SCN neurons that have elevated chloride reversal potentials due to the activity of a chloride transporter, NKCC1. Future studies will clarify which neurons are excited, at what times of day, and to what functional end.

Because chronic blockade of endogenous GABA signaling in the SCN raises the daytime peak in firing with little effect on the already-low nighttime firing in most neurons, it has been postulated that GABA plays an important role in governing

peak firing rates and enhancing sensitivity to depolarizing inputs (Aton et al. 2006). This is consistent with the evidence that GABA and its receptor agonists can modulate light-induced phase shifts in vivo and optic nerve input to the SCN in vitro (Gannon et al. 1995; Ehlen et al. 2008). Notably, daily GABA application can synchronize cultured SCN neurons (Liu and Reppert 2000). However, endogenous GABA signaling is not required for SCN neurons to synchronize to each other (Aton et al. 2006). Instead, GABA signaling from the ventral SCN acutely excites neurons in the dorsal SCN and from the dorsal SCN acutely inhibits neurons in the ventral SCN (Albus et al. 2005). This reciprocal, long-range, rapid synaptic communication may play a role in coordinating rhythms between the top and bottom of the SCN. We will benefit from further studies on the necessity of GABA signaling for SCN entrainment to environmental cues.

1.1.6 Other Signals Within the SCN

A number of other small molecules, primarily neuropeptides and cytokines, have been studied as intercellular signals in the SCN including prokineticin 2, neuromedin S and neuromedin U (Mori et al. 2005; Graham et al. 2005), met-enkephalin, angiotensin II (Brown et al. 2008), somatostatin (Ishikawa et al. 1997), and substance P (Kim et al. 2001). Cardiotrophin-like cytokine (CLC) falls into this category of signals of interest. Based on one report, approximately 1 % of SCN neurons synthesize CLC, and the genes encoding its receptor subunits (*Cntf*, *Gp130* and *Lifr*) are expressed along the third ventricle (Fig. 2) (Kraves and Weitz 2006). In vivo administration of CLC decreased running wheel activity in mice, whereas inhibition of GP130 increased locomotion without affecting the phase or period of circadian rhythms (Kraves and Weitz 2006). Similar results have been reported for Prokineticin 2 (Zhou and Cheng 2005), implicating them as humoral factors secreted by the SCN to regulate motor activity. In each case, these signals are made by a subset of dorsal SCN neurons, and their function within the SCN has yet to be elucidated.

2 Glia of the Circadian System

Although it is clear that much of daily rhythms in physiology and behavior arise from the activity of clock neurons (Nitabach and Taghert 2008; Hastings 1997), recent advances have revealed that the “other cells in the brain,” glia, also show circadian rhythms in vivo and in vitro. In 1993, Lavialle and Serviere discovered high-amplitude daily rhythms in the distribution of glial fibrillary acidic protein (GFAP) in astrocytes of the suprachiasmatic nucleus (SCN) (Lavialle and Serviere 1993). This rhythm persists in constant darkness in the SCN of hamsters, rats, and mice (Lavialle and Serviere 1993; Moriya et al. 2000), suggesting that this rhythm is intrinsic and independent of external light cues. The role of daily oscillations in

GFAP immunoreactivity on glial cells is unknown. Leone et al. suggest that oscillations in GFAP reflect a response of astrocytes in the SCN to inputs from the immune system. Moriya et al. speculate that GFAP plays a role in circadian rhythms in constant light conditions. Regardless, the conservation of the daily rhythms in GFAP distribution in the SCN among three mammalian species suggests it has some function.

Astrocytes communicate with nearby glia and neurons by releasing transmitter through a process known as gliotransmission (Perea et al. 2009; Fields and Burnstock 2006; Haydon 2001). The best known transmitters produced and released by astroglia are ATP, D-serine, and glutamate (Parpura and Zorec 2010). The mechanism of gliotransmission is thought to be dependent upon fluctuations in cytosolic calcium levels and vesicular release of transmitters.

The first (and only) direct demonstration that glial cells can modulate circadian physiology and behavior came from flies. In flies, the protein and mRNA levels of ebony, a glia-specific enzyme, are enriched around clock neurons and vary with time of day (Suh and Jackson 2007). Ebony is an *N*-beta-alanyl-biogenic amine synthetase capable of conjugating a beta-alanine to histamine, as well as other amine neurotransmitters (e.g., dopamine and serotonin). Mutants carrying any one of five *ebony* alleles show dramatic changes in the circadian period of their locomotor rhythms (Suh and Jackson 2007). This phenotype is rescued by glia-specific overexpression of *ebony* (Ng et al. 2011). These results led researchers to test whether glial signaling is required for circadian behaviors. They found that transgenic manipulation of the membrane potential, calcium signaling, or vesicular release in astroglia dramatically reduces the proportion of circadian flies and, interestingly, circadian rhythms in neuropeptide signaling (Ng et al. 2011).

In mammals, there is indirect evidence that glia contribute to circadian behaviors. GFAP knockout mice show longer periods of activity and more arrhythmicity in constant light conditions compared to wild type (Moriya et al. 2000). Manipulations of gliotransmission have been implicated in the regulation of sleep homeostasis, but not yet in circadian biology (Halassa and Haydon 2010). Here, we review the evidence for neural and glial control of glial circadian cycling in mammals.

2.1 Neuron-to-Glia Signaling

There is strong evidence for neuronal coordination of glial circadian rhythms in mammals. Glia cultured from mouse motor cortex with a knock-in bioluminescent reporter of Period2 expression show circadian rhythms that damp out over a week (Prolo et al. 2005). When cocultured with an SCN explant, glia express sustained circadian rhythms, suggesting that SCN neurons can coordinate glial rhythms through a diffusible signal (Prolo et al. 2005). In vivo, circadian rhythms in ATP release appear to derive primarily from astrocytes within the SCN (Womac et al. 2009). Interestingly, astrocytes in the SCN respond to photic stimulation with an

increase in cFOS expression (Bennett and Schwartz 1994), suggesting they may participate in the response to light and, perhaps, entrainment. Future work will likely focus on the role of glia in different aspects of circadian behavior and whether glia in different brain areas have different circadian functions.

2.1.1 VIP/VPAC2R

In addition to the well-established role of VIP in communication between circadian neurons (Vosko et al. 2007), VIP has been implicated in neuron-to-glia daily signaling. Cortical astrocytes respond to agonists for VPAC2R, but not VPAC1R (Zusev and Gozes 2004). Cultured astrocytes respond to nanomolar concentrations of VIP with clock gene induction, ATP release, and shifts in their circadian rhythms (Marpegan et al. 2009, 2011). Daily administration of VIP to cultured astrocytes sustains and entrains rhythmic circadian expression of Period2 (Marpegan et al. 2009). Gerhold and Wise have provided in vivo evidence for VIP-mediated circadian rhythms in glia. By suppressing expression of VIP in the SCN, they disrupted the diurnal rhythms in surface area observed in astrocytes that ensheathe gonadotropin-releasing hormone (GnRH) neurons (Gerhold and Wise 2006; Gerhold et al. 2005). They also showed mRNA expression of VPAC2 receptors in these astrocytes, indicating a direct interaction between VIP and glia (Gerhold and Wise 2006). This fluctuation in astrocyte surface area is thought to modulate stimulatory, neural inputs to GnRH neurons, thus modulating GnRH synthesis and release (Cashion et al. 2003). Further in vivo studies will be required to elucidate other potential roles of VIP in regulating astrocyte function in the SCN.

2.1.2 ATP/Purinergic Receptors

In addition to providing energy to cells, ATP acts as a transmitter to send signals to neighboring glial and neuronal cells in the nervous system (Haydon 2001; Suadicani et al. 2006). Extracellular accumulation of ATP in the SCN fluctuates in a circadian fashion, peaking in the middle of the night, or subjective night, in rats (Womac et al. 2009; Yamazaki et al. 1994). Cultured cortical astrocytes also display circadian rhythms in extracellular ATP accumulation (Womac et al. 2009; Burkeen et al. 2011; Marpegan et al. 2011). A circadian role for this extracellular ATP has not been identified.

ATP can act directly on purinergic receptors or be degraded into biologically active ADP or adenosine. The nucleoside adenosine has a predominantly inhibitory effect on neuronal activity in the CNS (Dunwiddie and Masino 2001). Adenosine has been implicated in the regulation of sleep (Chikahisa and Séi 2011). Adenosine receptor antagonists (i.e., caffeine) disrupt inhibitory effects caused by extracellular adenosine, leading indirectly to a stimulatory effect (Fredholm et al. 1999). In the retina, circadian changes in extracellular adenosine levels appear to arise from non-neuronal sources to regulate rod-cone coupling (Ribelayga et al. 2008). Thus,

ATP and other nucleosides can carry time-of-day information to regulate sensory processing.

2.2 *Glia-to-Neuron Signaling*

There is indirect evidence for astrocytes communicating circadian timing information to other cells. For example, because circadian changes in SCN ATP likely derive primarily from astrocytes, there is potential for glia to regulate SCN activity. Additionally, rhythms in excitatory amino acids (EAAs) including glutamate in the SCN may be due to astrocyte release (Shinohara et al. 2000). Evidence includes the few, if any, glutamatergic neurons in the isolated SCN, the calcium independence of EAA rhythms, and the persistent EAA rhythms in the presence of L-trans-pyrrolidine-2,4-dicarboxylic acid, a glutamate/aspartate uptake inhibitor (Shinohara et al. 2000). Because astrocytes can act as a source of calcium-independent neurotransmitter release (Malarkey and Parpura 2008), glia may regulate extracellular glutamate levels in the SCN. A recent study found that the circadian clock in cortical glia, however, does not regulate their glutamate reuptake on a daily basis (Beaulé et al. 2009). Future studies will likely focus on whether glia regulate EAA levels through circadian release.

3 Common Drugs and Their Effects on Circadian Signaling

This review has identified several signaling pathways involved in generation and regulation of daily rhythms in behavior and physiology. For example, neuropeptides within the SCN are involved in synchronizing circadian cells to each other, amplifying their daily cycling, and adjusting their rhythms to local time. Thus, drugs that impinge on neuropeptidergic receptors or signaling pathways can potently shape daily schedules. We must consider the likely effects of many drugs on the circadian timing system (see also Musiek and FitzGerald 2013; Antoch and Kondratov 2013; Ortiz-Tudela et al. 2013). One striking example is the drug most commonly taken by humans—caffeine. Caffeine adjusts circadian timing of electrical activity in the isolated SCN and of clock gene expression in cultured mammalian cells and modestly lengthens the circadian period of locomotor activity in mice (Oike et al. 2011; Wyatt et al. 2004; Ding et al. 1998). Despite an extensive literature on caffeine's effects on sleep and vigilance (Wright et al. 1997; Fredholm et al. 1999; Landolt et al. 1995), there have been no studies of the effects of caffeine on human circadian biology. Thus, the circadian effects of common pharmaceuticals are understudied and now can be easily assayed in vivo and in vitro.

We have stressed that some ligands have different effects at different times of day, for example, phase shifting the clock during the night, but not during the day.

This can arise from circadian changes in the abundance or activity of receptors or downstream, intracellular second messengers. In pharmacology, the consequences of this must be emphasized (see Musiek and FitzGerald 2013; Ortiz-Tudela et al. 2013). Time-dependent administration may determine the efficacy of a drug based on known circadian rhythms. For example, angiotensin II receptor antagonists, used in the treatment of hypertension, may be more effective at times when their target pathway is available for modulation (Portaluppi et al. 2012). Thus, chronopharmacology is the strategic use of drugs to affect the circadian clock or clock-regulated pathways.

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Part II
Circadian Control of Physiology and Behavior

Circadian Clocks and Metabolism

Biliana Marcheva, Kathryn M. Ramsey, Clara B. Peek, Alison Affinati, Eleonore Maury, and Joseph Bass

Abstract Circadian clocks maintain periodicity in internal cycles of behavior, physiology, and metabolism, enabling organisms to anticipate the 24-h rotation of the Earth. In mammals, circadian integration of metabolic systems optimizes energy harvesting and utilization across the light/dark cycle. Disruption of clock genes has recently been linked to sleep disorders and to the development of cardiometabolic disease. Conversely, aberrant nutrient signaling affects circadian rhythms of behavior. This chapter reviews the emerging relationship between the molecular clock and metabolic systems and examines evidence that circadian disruption exerts deleterious consequences on human health.

Keywords Circadian clock • Metabolism • Energy homeostasis • Metabolic disease • Nutrient sensing

1 Introduction

The daily order of temporal life is so innate as to slip from consciousness on most days. So it should not be surprising that a conceptual framework for studies of biological timing remains outside of the realm of modern medical practice. Yet with a transformation in our understanding of the molecular mechanism encoding circadian systems over the past 20 years, new studies have begun to bridge the gap from molecular clocks to human biology. Insight into circadian clocks and

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metabolism stems directly from the discovery that biological rhythms are sustained by a genetically encoded transcription network that functions as a molecular oscillator with near 24-h precision in most cell types, maintaining phase alignment in a range of behavioral, physiological, and biochemical processes with the environmental light cycle. The impact of circadian timing on human health has begun to emerge through observational population studies in individuals subjected to sleep restriction, shift work, and jet travel, in addition to experimental studies that reveal broad pathophysiologic consequences of circadian disruption on cognitive function, psychiatric disorders, cancer, metabolic syndrome, and inflammation (Bechtold et al. 2010; Reppert and Weaver 2002; Albrecht 2013). In this chapter, we focus on the growing body of evidence indicating a critical role of the clock network in metabolic homeostasis and highlight the cross talk between circadian and metabolic systems as a framework to understand effects of biological timing on physiology and disease states.

2 Clocks, Metabolism, and Disease

2.1 Rhythmicity of Metabolic Processes

While the most overt outputs of the mammalian clock are the sleep/wake and fasting/feeding cycles, the circadian clock also influences homeostasis across a broad range of behavioral and physiological processes, including glucose and lipid metabolism, body temperature, endocrine hormone secretion, and cardiovascular health (Fig. 1) (Panda et al. 2002b; Reppert and Weaver 2002). An evolutionary advantage of the circadian clock may be that it enhances energetic efficiency through temporal separation of anabolic and catabolic reactions (such as gluconeogenesis and glycolysis). An additional function of the clock is to maintain proper alignment of internal metabolic cycles relative to the sleep/wake cycle, enabling organisms to anticipate changes in the daily energetic environment tied to the rising and setting of the sun. In humans, circadian control over physiology has been well established through epidemiological research. For example, myocardial infarction, pulmonary edema, and hypertensive crises all have a tendency to peak at particular times during the day (Maron et al. 1994; Staels 2006). Circadian control of glucose metabolism has also been well documented, though the precise molecular mechanisms are not yet well understood. Glucose tolerance and insulin action are known to vary throughout the day, as oral glucose tolerance is impaired in the evening compared to morning hours due to combined effects of reduced insulin sensitivity and diminished insulin secretion in the nighttime. Glucose levels per se also display circadian oscillations and peak before the start of the active period (Arslanian et al. 1990; Bolli et al. 1984). Evidence from SCN-ablated rats and

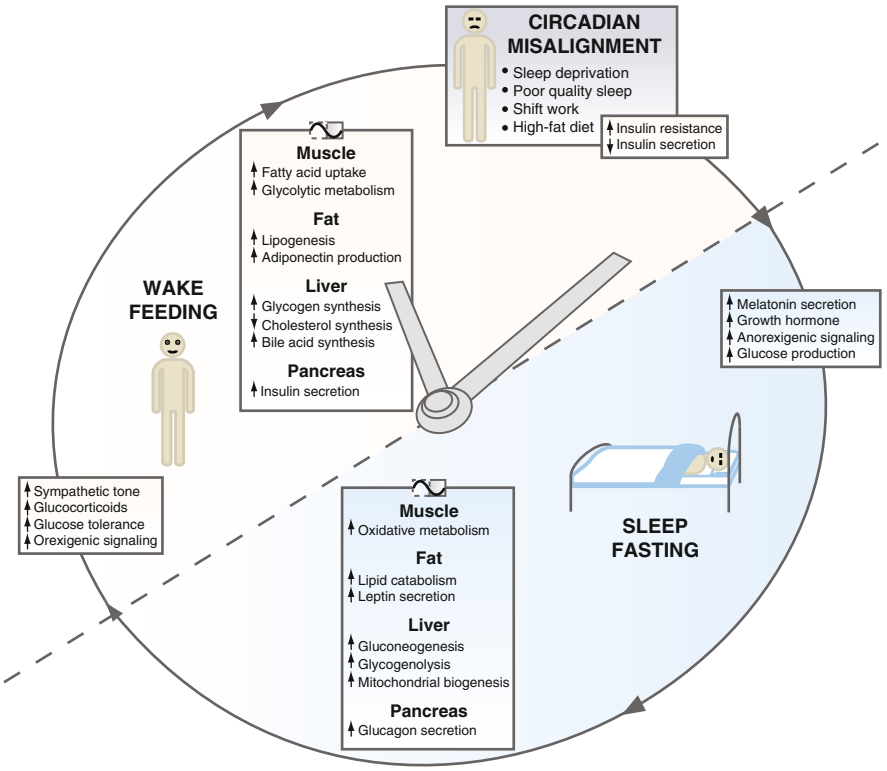

Fig. 1 Rhythmicity of metabolic processes according to time of day. The clock coordinates appropriate metabolic responses with the light/dark cycle and enhances energetic efficiency through temporal separation of anabolic and catabolic reactions in peripheral tissues. Circadian misalignment, which occurs during sleep disruption, shift work, and dietary alterations, disrupts the integration of circadian and metabolic systems, leading to adverse metabolic health effects [Figure modified from Bass and Takahashi (2010)]

degeneration of autonomic tracts linking SCN to liver further points toward a direct role for the circadian clock in glucose homeostasis, as these rats display loss of 24-h glucose rhythms (Cailotto et al. 2005; la Fleur et al. 2001). Of note, studies in both rodents and humans suggest that loss of circadian rhythmicity of glucose metabolism may even contribute to the development of metabolic disorders such as type 2 diabetes, as rhythms of insulin secretion, glucose tolerance, and corticosterone release are diminished in streptozotocin-induced diabetic rats and in patients with type 2 diabetes (Oster et al. 1988; Shimomura et al. 1990; Van Cauter et al. 1997). Gaining a better understanding of the molecular mechanisms underlying circadian control of glucose homeostasis and other physiological processes will therefore be critical for enabling temporal evaluation in the diagnosis and treatment of metabolic disorders.

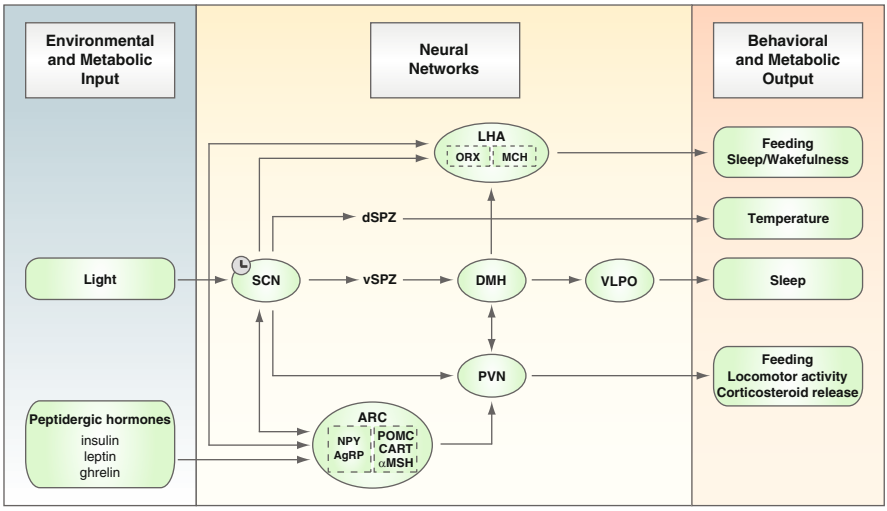

Fig. 2 Neural circuits linking hypothalamic regions important in circadian and energetic control. Signals from the exogenous environment (i.e., light) and endogenous metabolism (i.e., hormones and metabolites) are integrated in a network of hypothalamic neuronal centers (see text for details), which in turn impart rhythmicity on behavioral and metabolic outputs, including sleep, feeding and activity behavior, thermogenesis, and hormone secretion [Figure modified from Huang et al. (2011)]

2.2 *CNS Circuits Integrating Circadian and Metabolic Processes*

Circadian and metabolic processes interact at both the neuroanatomic and neuroendocrine levels to regulate overall metabolic homeostasis. In order to better appreciate how the circadian clock network within brain regulates whole body metabolism, it is important to understand the anatomic connections between brain centers essential for circadian rhythmicity and those that control appetite and energy expenditure (Fig. 2) (reviewed in Horvath 2005; Horvath and Gao 2005; Saper et al. 2005; Slat et al. 2013; Kalsbeek and Fliers 2013). Classical lesioning studies performed in the 1970s first determined that the "master pacemaker" neurons are localized to the suprachiasmatic nuclei (SCN), which consist of bilateral nuclei within the anterior hypothalamus that receive environmental light input via the retinohypothalamic tract (RHT). SCN ablation abolishes 24-h rhythms of locomotor activity, feeding, drinking, and sleep. Subsequent studies in the 1990s revealed that transplantation of a short-period (*tau*) mutant SCN into an SCN-lesioned wild-type hamster results in the wild-type hamster having a period length identical to that of the *tau* mutant donor SCN (Ralph et al. 1990). However, 24-h rhythms of glucocorticoid and melatonin oscillations were not restored. These experiments established that direct neuronal projections, in addition to secreted factors, are necessary for SCN regulation of behavioral and metabolic homeostasis (Cheng et al. 2002; Kramer et al. 2001; Meyer-Bernstein et al. 1999; Silver et al. 1996).

Elegant tracing studies have revealed that neurons from the SCN predominantly synapse directly on cell bodies within the ventral and dorsal subparaventricular zones (vSPZ and dSPZ, respectively) and the dorsomedial hypothalamus (DMH). vSPZ neurons are involved in regulation of sleep/wake and activity cycles, but not body temperature cycles, while neurons within the dSPZ control temperature rhythms and have minimal impact on sleep and activity cycles (Lu et al. 2001). The SPZ also projects to the DMH, a brain region important for rhythms of the sleep/wake cycle, locomotor activity, body temperature, food intake, and corticosteroid secretion (Chou et al. 2003). Projections from neurons within the DMH target neuronal centers involved in the regulation of sleep/wake cycle (ventrolateral preoptic nucleus—VLPO), corticosteroid release (paraventricular nucleus—PVN), and feeding and wakefulness (lateral hypothalamus—LHA). As such, the DMH acts as a relay center, amplifying circadian signals from the SCN to multiple regions of the brain involved in regulation of sleep, activity, and feeding.

The DMH and LHA also receive input from the arcuate nucleus (ARC), which plays a well-characterized role in the regulation of feeding and appetite. The ARC contains orexigenic neurons that express neuropeptide Y (NPY) and the agouti-related protein (AgRP), as well as neurons expressing anorexigenic peptides including pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). Because the choroid plexus allows passage through the blood–brain barrier, the ARC is uniquely positioned to integrate humoral signals from the periphery with neuronal signals within the hypothalamus. For example, leptin, a hormone secreted in proportion to fat mass by adipose tissue, stimulates production of α-melanocyte-stimulating hormone (α-MSH) from POMC/CART neurons while simultaneously inhibiting NPY/AgRP production within the ARC (Cowley et al. 2001; Frederich et al. 1995). This in turn decreases production of the orexigenic peptides orexin (ORX) and melanin-concentrating hormone (MCH) within the LHA and suppresses appetite and food intake. When leptin levels are low, the orexigenic neurons in the ARC produce NPY and AgRP, which stimulate hunger and decrease energy expenditure via signaling to the LHA. Interestingly, in addition to regulation by nutrient status, leptin also displays a circadian pattern of expression. While our knowledge of circadian regulation of circulating hormones from the periphery is still quite incomplete, it will ultimately be important to identify how signals regulated by the nutritional status of the organism communicate with and co-regulate brain centers involved in control of activity, feeding, sleep, and metabolism. In this regard it is of note that NPY, AgRP, and orexin display circadian patterns of expression within the hypothalamus, with peaks around the beginning of the active period, while α-MSH levels are highest at the beginning of the inactive period (Kalra et al. 1999; Lu et al. 2002). Thus, understanding the neural networks integrating centers involved in regulation of circadian rhythms, sleep, and energy homeostasis may shed light on the interplay between anticipatory and adaptive behaviors involved in long-term energy constancy.

2.3 *Peripheral Oscillators and Circadian Regulation of Metabolic Transcription Networks*

Prior to the discovery of molecular clock genes, a prevailing model held that circadian rhythms represent a unique property of pacemaker neurons. However, seminal experiments performed in the 1990s established the presence of cell-autonomous circadian gene rhythmic expression in cultured fibroblasts, demonstrating the ubiquity of circadian transcriptional oscillators throughout all cells (Balsalobre et al. 1998). Subsequent molecular analyses have revealed that the clock network is indeed expressed not only in the SCN, but in most mammalian tissues, including those essential for cardiometabolic function, such as liver, pancreas, muscle, and heart (Davidson et al. 2005; Marcheva et al. 2010; Wilsbacher et al. 2002; Yamazaki et al. 2000; Yoo et al. 2004; reviewed in Brown and Azzi 2013). Because the phase of peripheral clocks is delayed compared to that of the SCN, and since ablation of the SCN abolishes synchrony of peripheral oscillators, it is believed that the SCN functions as a master pacemaker maintaining phase alignment of autonomous cellular clocks throughout all peripheral tissues (Balsalobre et al. 1998; Sakamoto et al. 1998).

Emerging genomic studies have illuminated the multifaceted function of peripheral circadian oscillators at the cellular level (Fig. 3). For instance, transcriptional profiling studies have revealed that ~10 % of all mammalian genes across multiple tissues exhibit 24-h variations in mRNA levels (Miller et al. 2007; Oishi et al. 2003; Panda et al. 2002a; Rey et al. 2011; Rudic et al. 2005; Storch et al. 2002; Zvonic et al. 2006). Importantly, gene ontogeny analyses have shown that many of these rhythmic genes cluster within classes regulating intermediary metabolism, including processes such as mitochondrial oxidative phosphorylation, carbohydrate metabolism and transport, lipid biosynthesis, adipocyte differentiation, and cholesterol synthesis and degradation (Bass and Takahashi 2010; Delaunay and Laudet 2002; Doherty and Kay 2010; Panda et al. 2002a; Yang et al. 2006). While only a small subset of these oscillating metabolic genes is a direct target of the molecular clock, many encode transcription factors, transcription or translation modulators, or rate-limiting enzymes, which in turn impart rhythmicity on downstream metabolic genes and processes (Noshiro et al. 2007; Panda et al. 2002a; Ripperger and Schibler 2006). Interestingly, the phase of oscillation and the level of expression of each metabolic gene vary across different tissues, suggesting that the circadian system responds to both local and systemic cues to control diverse metabolic processes in a physiologically meaningful manner (Delaunay and Laudet 2002; Kornmann et al. 2007). Not surprisingly, mutation of the core molecular clock disrupts the rhythmic expression of numerous key metabolic genes (Lamia et al. 2008; McCarthy et al. 2007; Oishi et al. 2003; Panda et al. 2002a). Whether rhythmicity of these metabolic genes is secondary to the feeding rhythm or arises due to intrinsic clock expression within the periphery has been a long-standing question. Only recently, studies involving tissue-specific circadian gene mutant mice have indicated that molecular clocks in the periphery play a crucial role in

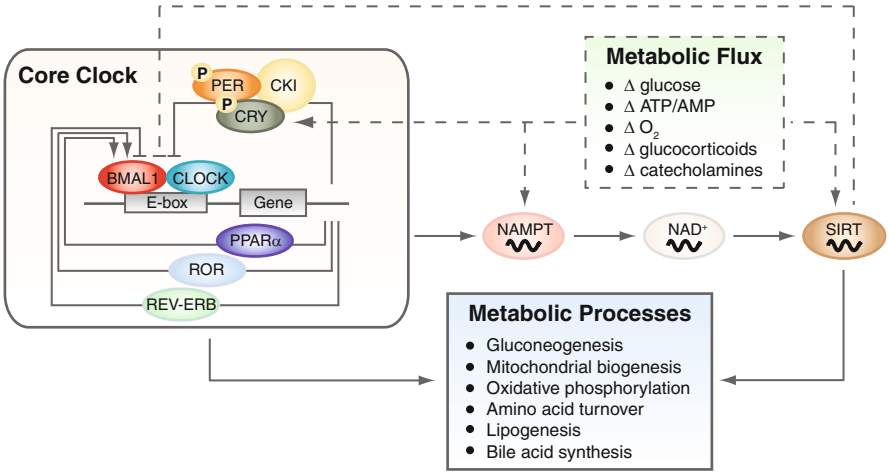

Fig. 3 Cross talk between the core clock mechanism and metabolic pathways. The core clock consists of a series of transcription/translation feedback loops that either directly or indirectly synchronize diverse metabolic processes. The clock also receives reciprocal input from nutrient signaling pathways (including NAD^+-dependent sirtuins), which function as rheostats to coordinate metabolic processes with daily cycles of sleep/wakefulness and fasting/feeding [Figure modified from Bass and Takahashi (2010)]

imparting rhythmicity to metabolic gene oscillation (Lamia et al. 2008; Marcheva et al. 2010; Sadacca et al. 2010).

Although the mammalian core clock genes are well defined, the precise role of both central and peripheral oscillators in the maintenance of energy balance and metabolic homeostasis is still not well understood. Research aiming to elucidate the molecular pathways linking the circadian clock with metabolic sensors remains an active area of investigation.

3 Circadian Disruption and Disease

3.1 Metabolic Phenotypes of Circadian Mutant Mice

Mouse models have been invaluable in defining the roles of individual core clock genes in the generation and maintenance of circadian rhythmicity and have recently begun to provide insight into the metabolic functions of the circadian clock. The first genetic link between circadian rhythmicity and metabolism was discovered in mice carrying the $Clock^{\Delta 19/\Delta 19}$ mutation. While initial studies found that these animals become arrhythmic when subjected to total darkness, it was subsequently observed that they also display attenuated diurnal feeding rhythms, hyperphagia, hyperlipidemia, hyperleptinemia, hepatic steatosis, and hyperglycemic hypoinsulinemia due

to impaired insulin secretion and islet proliferation (Marcheva et al. 2010; Turek et al. 2005). In addition, $Clock^{\Delta 19/\Delta 19}$ animals exhibit loss of rhythmic expression of key metabolic and proliferative genes in liver, muscle, and pancreas, which undoubtedly contributes to the extensive disruption of glucose and lipid homeostasis (Marcheva et al. 2010; McCarthy et al. 2007; Miller et al. 2007; Panda et al. 2002a). Knockout of *Clock* also compromises renal sodium reabsorption and reduces arterial blood pressure (Zuber et al. 2009). Furthermore, overexpression of the $Clock^{\Delta 19}$ allele in cardiomyocytes alters heart rate variability, contractility, and responsiveness to changes in afterload, revealing a role of the peripheral circadian gene network in the control of cardiac fuel handling (Bray et al. 2008).

Studies in mice mutant for BMAL1, the heterodimeric partner of CLOCK, have revealed that, in addition to causing arrhythmic behavior, loss of BMAL1 also impairs adipogenesis, adipocyte differentiation, and hepatic carbohydrate metabolism (Lamia et al. 2008; Rudic et al. 2004; Shimba et al. 2005). Mutation of *Bmal1* also leads to disruption of circadian variation in blood pressure and heart rate, to increased susceptibility to vascular injury, and to skeletal muscle pathologies (Anea et al. 2009; Curtis et al. 2007; McCarthy et al. 2007). Various peripheral tissue-specific *Bmal1* knockout mouse models, which exhibit normal circadian activity and feeding rhythms, have provided further insight into the role of the cell-autonomous clock in metabolism and energy balance. For example, pancreas-specific *Bmal1* disruption leads to hyperglycemia, impaired glucose tolerance, and decreased insulin response due to impaired β-cell proliferation and insulin granule exocytosis, while liver-specific *Bmal1* deletion leads to loss of oscillation of key hepatic metabolic genes, impaired gluconeogenesis, exaggerated glucose clearance, and hypoglycemia during the resting phase (Lamia et al. 2008; Marcheva et al. 2010; Sadacca et al. 2010). Thus, tissue-specific circadian clocks have distinct roles within pancreatic islets and liver, affecting opposing metabolic processes and thereby contributing to glucose constancy across periods of feeding and fasting.

Genetic loss of core clock genes downstream of the CLOCK/BMAL1 heterodimer also leads to metabolic abnormalities. Disruption of both *Cry1* and *Cry2* in mice results in glucose intolerance, elevated corticosterone levels, increased glucocorticoid transactivation in liver, altered lipogenic and steroidogenic pathways, and impaired body growth and liver regeneration (Bur et al. 2009; Lamia et al. 2011; Matsuo et al. 2003; Okamura et al. 2011). Knockdown of *Cry1* and *Cry2* was also found to increase expression of gluconeogenic genes and to augment hepatic glucose production (Zhang et al. 2010). These observations are consistent with findings that adenoviral overexpression of CRY1 decreases fasting glucose levels and improves insulin sensitivity in insulin-resistant $Lepr^{db/db}$ mice, while overexpression of mutant CRY1 results in polydipsia, polyuria, and hyperglycemia, all symptoms of diabetes mellitus (Okano et al. 2009; Zhang et al. 2010). Deficiency of PER2, another member of the core circadian feedback loop, abolishes glucocorticoid rhythmicity and protects mice from development of glucose intolerance in response to glucocorticoids (So et al. 2009; Yang et al. 2009). Finally,

disruption of the ability of the circadian kinase *CK1ε* to phosphorylate its PER and CRY protein targets (*tau* mutation of the Syrian hamsters) is also associated with reduced growth and elevated metabolic rate (Lucas et al. 2000; Oklejewicz et al. 1997).

Mice carrying mutations of nuclear hormone receptors (NHRs) that participate in circadian transcription feedback loops also display alterations in metabolic function. For example, in addition to a shorter circadian period in constant darkness, mice lacking the *Bmal1* repressor REV-ERBα exhibit altered lipid and bile metabolism (Le Martelot et al. 2009; Preitner et al. 2002). Conditional liver-specific overexpression of *Rev-erbα* induces changes in expression of genes involved in xenobiotic detoxification, carbohydrate and energy metabolism, and lipid and sterol homeostasis (Kornmann et al. 2007). Deficiency of the *Bmal1* activator RORα in *staggerer* mice leads to predisposition to age-related phenotypes such as atherosclerosis (Akashi and Takumi 2005; Mamontova et al. 1998; Sato et al. 2004). Similarly, vascular deletion of *Pparγ*, a stimulator of *Bmal1* expression, causes a significant reduction of the daily fluctuation in heart rate and blood pressure, modifies the diurnal variation in sympathetic nerve activity, and alters the expression of vascular adrenoceptors (Berger 2005; Wang et al. 2008). Additionally, ablation of *Pgc-1α*, a rhythmically expressed metabolic regulator and coactivator of the RAR-related orphan receptor (ROR) family of orphan nuclear receptors, leads to abnormal rhythms of locomotor activity, body temperature, and metabolic rate in mice (Liu et al. 2007).

Finally, disruption in the expression of clock-controlled genes downstream of the core circadian network also impacts metabolism. For instance, mice mutant for the circadian poly(A) deadenylase *Nocturnin*, which is involved in posttranscriptional regulation of rhythmic gene expression, exhibit alterations in glucose tolerance and peripheral tissue insulin sensitivity. They are also resistant to diet-induced obesity and hepatic steatosis due to defective lipid absorption in the small intestine (Douris et al. 2011; Green et al. 2007). Similarly, disruption of the estrogen-related receptor-α (ERR-α), another orphan nuclear receptor, has been implicated in resistance to high-fat diet and metabolic dysregulation, including reduced peripheral fat deposits, hypoglycemia, and time-dependent hypoinsulinemia (Dufour et al. 2011). Of note, ablation of ERR-α also modifies locomotor activity rhythms and the expression patterns of the core clock genes, suggesting a function of ERR-α as a potential regulator of the circadian clock (Dufour et al. 2011). Collectively, these animal model studies have unveiled the importance of both central and peripheral circadian clocks for the maintenance of energy homeostasis.

3.2 Circadian Gene Polymorphisms and Metabolic Phenotypes in Humans

In addition to the recent findings in animal models, mounting evidence suggests that genetic variation in circadian genes also influences metabolic parameters in

humans. A number of genome-wide association studies (GWAS) have uncovered links between polymorphisms in *CLOCK* and susceptibility to hypertension, obesity, and metabolic syndrome (Garaulet et al. 2009; Sookoian et al. 2008, 2010). Single-nucleotide polymorphisms (SNPs) in *CLOCK* are also associated with high plasma ghrelin concentrations, short sleep duration, and altered eating behaviors, leading to higher total energy intake, decreased compliance with prescribed diet plans, and, ultimately, resistance to weight loss (Garaulet et al. 2010b, 2011). Case–control studies have further revealed a correlation between common genetic variations of *CLOCK* and the incidence and severity of nonalcoholic fatty liver disease (NAFLD), one of the most common disorders observed in obese persons (Sookoian et al. 2007).

Genetic variants in *BMAL1* have also been linked with the development of hypertension and type 2 diabetes, and SNPs in loci in or near *CRY2* have been associated with fasting glucose concentrations (Dupuis et al. 2010; Woon et al. 2007). *PER2* polymorphisms have also been linked with hyperglycemia, abdominal obesity, and unhealthy feeding behavior phenotypes, while expression of *PER2* mRNA may also correlate with metabolic markers in humans (Ando et al. 2010; Englund et al. 2009; Garaulet et al. 2010a; Gomez-Abellan et al. 2008). Conversely, a rare variant of *NAMPT1*, a gene involved in a clock negative feedback loop, is associated with protection from obesity (Blakemore et al. 2009).

GWAS studies have also recently indicated that MTNR1A and MTNR1B, G-protein-coupled receptors for melatonin, a circadianly regulated circulating hormone implicated in the regulation of sleep and glucose homeostasis, are associated with high fasting plasma glucose levels (Dubocovich et al. 2003; Li et al. 2011; Ling et al. 2011; Peschke and Muhlbauer 2010; Ronn et al. 2009; Takeuchi et al. 2010; Tam et al. 2010). Genetic variants in and near *MTNR1B* are further associated with impaired insulin secretion and increased risk of developing type 2 and gestational diabetes mellitus, while the SNP rs2119882 in the *MTNR1A* gene is linked to insulin resistance and susceptibility to polycystic ovary syndrome, a hormonal disorder in women often associated with obesity, type 2 diabetes, and heart disease (Kim et al. 2011; Kwak et al. 2012; Li et al. 2011; Lyssenko et al. 2009; Staiger et al. 2008).

While most GWAS analyses do not take into account the added complexity of gene–gene interactions, one study demonstrated the existence of a synergistic effect of specific polymorphisms in *PER2*, *PER3*, *CLOCK*, and *BMAL1* that accounts for morning or evening activity preference in humans (Pedrazzoli et al. 2010). The extensive cross talk between circadian and metabolic networks further adds to the complexity of analysis of human genetic studies. For instance, environmental dysregulation of metabolic homeostasis, such as overnutrition with western diet, may reciprocally feedback to impair circadian homeostasis (Kohsaka et al. 2007). Further, carriers of the *CLOCK* SNP rs4580704 display lower glucose levels and improved insulin sensitivity only when on a diet high in monounsaturated fatty acids, while an association between the *CLOCK* SNP rs1801260 and increased waist circumference is evident only in the presence of saturated fatty acids (Garaulet et al. 2009). A complete understanding of circadian gene function in

humans will ultimately require consideration of both genetic and nutritional variables. As new evidence for interactions between clocks and metabolism emerges from epidemiologic and association studies, it underscores the clinical importance of rhythmicity for the maintenance of metabolic homeostasis and emphasizes the significance and implications of a better understanding of the interconnections between the two systems.

3.3 Impact of Circadian Misalignment in Humans

Since the introduction of artificial light and nighttime work, serious health consequences have been reported for those who sleep less and/or routinely disconnect their working time from the light/dark cycle. Reduced sleep duration (both acute and chronic) and poor-quality sleep are linked with impaired glucose tolerance, reduced insulin responsiveness following glucose challenge, increased body mass index, decreased levels of leptin, and increased levels of ghrelin (Donga et al. 2010; Gottlieb et al. 2005; Knutson and Van Cauter 2008; Megirian et al. 1998; Nilsson et al. 2004; Spiegel et al. 2009; Taheri et al. 2004). Association studies have further revealed that shift workers have increased risk of obesity, metabolic dysfunction, cardiovascular disease, cancer, and ischemic stroke (Di Lorenzo et al. 2003; Ellingsen et al. 2007; Karlsson et al. 2001, 2003). Social jetlag, the discrepancy between the circadian and social clock resulting in chronic sleep loss, has also been linked to increased BMI (Roenneberg et al. 2012). One of the most compelling clinical studies to examine the role of circadian alignment on metabolic physiology comes from an experimental paradigm in which healthy volunteers were placed on a 28-h "day" and scheduled to sleep at different phases throughout the circadian cycle. When the subjects were shifted 12 h from their normal sleep/wake cycle, they exhibited decreased leptin, increased glucose, and elevated blood pressure. In addition, their post-meal glucose response was similar to that seen in prediabetic patients (Scheer et al. 2009). Together, these studies highlight the detrimental health effects of disruption of the circadian system and the importance of synchronization of physiological systems with the light/dark cycle for maintenance of overall health.

4 Clocks and Nutrient Sensing

4.1 Impact of Diet Composition and Feeding Time

The aforementioned trend in modern society to disrupt the traditional sleep/wake cycle is coupled with a tendency to eat at irregular times. However, high-energy food intake in the evening and fasting in the morning have both been associated

with the development of obesity, and skipping breakfast has also been shown to impair postprandial insulin sensitivity and fasting lipid levels in humans (Ekmekcioglu and Touitou 2011; Farshchi et al. 2005). Further, mice fed a high-fat diet (HFD) have increased daytime activity, lengthened period of locomotor activity rhythms, and altered expression of clock and clock-controlled genes involved in fuel utilization (Kohsaka et al. 2007). Interestingly, these mice consume nearly all of their extra calories during the 12-h light phase, suggesting that feeding at the incorrect time in the light/dark cycle (i.e., their rest period) exacerbates the obesogenic effects of high caloric intake due to desynchronization of various behavioral, hormonal, and molecular rhythms involved in maintaining energy balance (Kohsaka et al. 2007). In agreement with these observations, food restriction to the active (dark) phase in genetically obese mice with disrupted diurnal feeding patterns leads to improvement of their obesity and metabolic disorders, while HFD consumption exclusively during the rest (light) phase in wild-type mice significantly contributes to weight gain (Arble et al. 2009; Masaki et al. 2004). Together, these data suggest that the normal alignment of feeding and activity with the environmental light cycle is critical for the maintenance of energy homeostasis, though further studies will be necessary to understand the precise mechanisms of how the timing of food intake impacts energy constancy.

Restricted feeding (RF) (i.e., limiting food availability to the normal rest period) in rodents also induces a burst of food anticipatory activity (FAA), or an increase in locomotor activity prior to mealtime. On a molecular level, RF entrains circadian oscillations in peripheral tissues, such as liver and kidney, without affecting the clock rhythms in the central pacemaker in the SCN, thereby uncoupling the phase of peripheral clocks from that of the SCN (Damiola et al. 2000; Stokkan et al. 2001). However, the involvement of circadian oscillators in FAA remains controversial because while lesioning of the dorsomedial nucleus alters FAA, food anticipatory behavior persists in *Bmal1* nullizygous mice (Fulton et al. 2006; Mieda et al. 2006). It is possible that the FAA constitutes a metabolic oscillator responsive to peripheral, neural, or circulating signals elicited by food ingestion. Resolution of the precise stimuli and neural pathways involved in FAA and understanding the effect of these nutrient signaling pathways on core properties of the SCN pacemaker remain important avenues for further investigation.

In addition to timing of food availability affecting the circadian outputs of the clock, caloric restriction (i.e., restriction of the total number of calories consumed without malnutrition) induces phase advances in rat behavioral and physiological circadian rhythms and alters expression of clock genes and neuropeptides in the mouse SCN (for review, Challet 2010). Prolonged fasting also advances the phase of free-running rhythms of locomotor activity and temperature (Challet et al. 1997). Together, these studies demonstrate that feeding behavior plays an essential role in coordinating the circadian rhythms of metabolism, though the precise identity of the signals that are able to reset the clock remains obscure.

4.2 Circadian Control of NAD^+ Biosynthesis and Sirtuin/PARP Activity

One potential molecule that has been implicated as a mediator between circadian and metabolic pathways is nicotinamide adenine dinucleotide NAD^+, a key cofactor involved in cellular redox reactions. The molecular clock directly regulates transcription of nicotinamide phosphoribosyltransferase (NAMPT), a key rate-limiting enzyme in the NAD^+ salvage pathway (Nakahata et al. 2009; Ramsey et al. 2009). Consistent with circadian regulation of NAMPT, NAD^+ levels also oscillate in peripheral tissues such as liver and adipose tissue, even when animals are maintained in constant darkness (Ramsey et al. 2009; Sahar et al. 2011). Importantly, mice with mutations in the activator genes *Clock* and *Bmal1* exhibit constitutively low NAD^+ levels, while those deficient in the clock repressor genes *Cry1* and *Cry2* have elevated NAD^+, indicating direct regulation of NAD^+ by the clock (Ramsey et al. 2009).

In addition to its role in redox reactions, NAD^+ also acts as a cofactor for several enzymatic reactions, including NAD^+-dependent deacetylation and ADP-ribosylation. The circadian clock was recently shown to modulate the activity of the metabolic enzyme, SIRT1, a class III protein deacetylase and a member of the sirtuin family of NAD^+-dependent deacetylases. SIRT1 resides primarily in the nucleus and targets several transcription factors involved in the maintenance of nutrient flux, including Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha (PGC-1α), Forkhead Box Protein O1 (FOXO1), Transducer of Regulated CREB Protein 2 (TORC2), Sterol Regulatory Element-Binding Protein 1c (SREBP-1c), and Signal Transducer and Activator of Transcription 3 (STAT3) (Haigis and Sinclair 2010; Rodgers et al. 2005; Sahar et al. 2011; Saunders and Verdin 2007). SIRT1 is a critical regulator of metabolic processes such as gluconeogenesis, lipid metabolism, and insulin sensitivity, as well as lifespan (Haigis and Sinclair 2010; Sahar et al. 2011; Saunders and Verdin 2007). Through rhythmic NAD^+ biosynthesis, the circadian clock modulates SIRT1 activity, which then coordinates the daily transitions between the periods of fasting and feeding. Of note, SIRT1 also modulates CLOCK/BMAL1 activity, generating a negative feedback loop in which circadian control of NAD^+-mediated sirtuin activity in turn regulates the clock itself through interaction with PER2, CLOCK, and BMAL1 (Asher et al. 2008; Grimaldi et al. 2009; Nakahata et al. 2008). Thus, the cross talk between the biological clock and the NAMPT/NAD^+/SIRT1 pathway provides a nexus linking the circadian system and nutrient-sensing pathways.

It will be of great interest to determine whether circadian control of NAD^+ biosynthesis affects other NAD^+-dependent metabolic enzymes, including the six other mammalian sirtuin homologs (SIRT2–7) or the poly (ADP-ribose) polymerase (PARP-1). SIRT3–5 are primarily localized to mitochondria and have recently been characterized as important regulators of oxidative (fasting) metabolic pathways, including fatty acid oxidation, TCA cycle, ketogenesis, urea cycle, and oxidative phosphorylation (Haigis et al. 2006; Hallows et al. 2006, 2011; Hirschey et al. 2010;

Huang et al. 2010; Nakagawa and Guarente 2009; Schwer et al. 2006; Shimazu et al. 2010; Someya et al. 2010). While little is known about circadian rhythms of NAD^+ in mitochondria, it is intriguing to speculate that the circadian clock may influence mitochondrial sirtuins. PARP-1, which also plays an important role in the response to metabolic stress, has recently been identified as a regulator of clock gene expression through direct ADP-ribosylation of CLOCK and inhibition of CLOCK/BMAL1 DNA-binding activity (Asher et al. 2010). Interestingly, PARP-1 activity in mice is rhythmic, suggesting circadian control of ADP-ribosylation (Asher et al. 2010; Kumar and Takahashi 2010). However, at present, there is no evidence to suggest that these oscillations are mediated by clock-dependent control of NAD^+ biosynthesis.

4.3 A Role of Redox in Circadian Rhythms

Prior to the discovery that the molecular clock directly regulates rhythms of NAD^+, in vitro studies by McKnight and coworkers revealed that cellular redox status affects the circadian clock. Increased levels of oxidized cofactors (NAD^+ or $NADP^+$) decrease the ability of CLOCK/BMAL1 and NPAS2/BMAL1 to bind to DNA in purified systems, suggesting that cellular redox changes may be sufficient to entrain clocks (Rutter et al. 2001).

Interestingly, recent studies have further identified ~24-h rhythms in the cellular redox state, which control circadian oscillations of the oxidation state of the peroxiredoxin family of antioxidant enzymes (Kil et al. 2012; O'Neill and Reddy 2011; O'Neill et al. 2011; Vogel 2011). Oscillating peroxiredoxin redox state affects its antioxidant activity, generating self-sustained rhythms of cellular redox status even in the absence of transcriptional control of circadian gene expression (both in anucleate human red blood cells and in single-celled alga *Ostreococcus tauri* treated with inhibitors of transcription and translation) (O'Neill and Reddy 2011; O'Neill et al. 2011). More recent studies have revealed that rhythms of peroxiredoxin oxidation exist in all domains of life and that these rhythms persist even in the presence of genetic clock disruption in mammalian cells, fungi and flies (Edgar et al. 2012; O'Neill and Reddy 2011). These studies raise the possibility that oscillations in cellular redox state may be an integral mechanism by which circadian rhythms of metabolic processes are controlled and that these rhythms may be maintained independently from the molecular clock transcriptional feedback loop.

4.4 A Link Between Circadian Rhythms and Nuclear Hormone Receptor Pathways

Several recent studies have focused on the role of nuclear hormone receptors as potential nutrient-sensing factors linking metabolic and circadian pathways. NHRs

comprise a large family of proteins, containing both DNA- and ligand-binding domains that regulate their activities as transcriptional activators and/or repressors. Known NHR ligands include a wide range of molecules (e.g., steroid hormones, fatty acids, heme, sterols), though many NHRs are still classified as "orphan" since their endogenous ligands have not yet been identified (Sonoda et al. 2008). Interestingly, more than half of the ~50 known NHRs display rhythmic expression patterns in peripheral tissues and are thus attractive candidates as integrators of circadian and nutrient-sensing pathways (Asher and Schibler 2011; Teboul et al. 2009; Yang et al. 2006, 2007).

Some NHRs, such as members of the REV-ERB and ROR families, are both transcriptional targets of the CLOCK/BMAL1 complex as well as transcriptional regulators of clock genes themselves. In the SCN and most metabolic tissues, REV-ERBs repress and RORs activate *Bmal1* transcription, generating a short negative feedback loop (Akashi and Takumi 2005; Duez and Staels 2009; Preitner et al. 2002; Sato et al. 2004) (refer also to Sect. 3.1). As sensors of metabolites including heme, fatty acids, and sterols, REV-ERBα/β and RORα integrate nutrient signals with transcriptional regulation of the clock (Jetten 2009; Kallen et al. 2004; Yin et al. 2007). REV-ERBs and RORs also directly interact with other important metabolic factors, including the transcriptional regulator PGC-1α, which coactivates *Bmal1* with RORα and β and also plays a key role in regulation of mitochondrial oxidative metabolism (Grimaldi and Sassone-Corsi 2007; Liu et al. 2007). Interestingly, activity of PGC-1α is regulated by SIRT1-mediated NAD^+-dependent deacetylation, suggesting that circadian regulation of NAD^+ may represent yet another metabolic feedback loop involving PGC-1α (Rodgers et al. 2005).

In addition to REV-ERBs and RORs, other NHRs are important for the coordination of molecular clocks and nutrient-sensing pathways. For example, members of the PPAR family of NHRs are also regulators of clock gene expression. PPARα activates both *Bmal1* and *Rev-erbα* in liver (Li and Lin 2009; Schmutz et al. 2010; Yang et al. 2007). Conversely, several proteins, including PPARα, γ, and δ, are transcriptionally regulated by the circadian clock (Li and Lin 2009). Ligands for PPARs include various types of lipids, including the circulating gut metabolite oleylethanolamide (OEA), which is generated and released from the small intestine and suppresses food intake during the rest period in a PPARα-dependent manner (Fu et al. 2003; Rodriguez de Fonseca et al. 2001). In addition, PPARγ maintains daily rhythms of blood pressure and heart rate in blood vessels via regulation of *Bmal1* expression (Wang et al. 2008). Further studies will be necessary to determine the importance of other lipid-derived PPAR ligands as effectors of the molecular clock.

The glucocorticoid receptor (GR) is another important NHR involved in the cross talk between central and peripheral circadian clocks. GR is expressed in many metabolic tissues, including liver, skeletal muscle, heart, and kidney, and activates several metabolic pathways including lipid metabolism and gluconeogenesis (Dickmeis and Foulkes 2011). GR is activated by glucocorticoid (GC) steroid hormones, which are produced and secreted in a circadian manner from the adrenal cortex. Timed release of GC from the adrenal cortex is generated through relays

from the SCN to the hypothalamic-pituitary-adrenal axis (HPA). As such, GC rhythms are entrained by light and peak in the early morning in humans (Chung et al. 2011; Oster et al. 2006). Rhythmic GC release affects GR activity in peripheral tissues and thereby acts to synchronize peripheral clocks with the SCN (Teboul et al. 2009). Indeed, liver-specific GR knockout mice display accelerated clock phase-shifting in response to daytime food restriction, suggesting a lack of entrainment by the central clock (Le Minh et al. 2001). Furthermore, pharmacological activation of GR by dexamethasone resets the peripheral clock in liver, heart, and kidney, presumably by direct GR regulation of *Rev-erbα* and *Per1* expression (Balsalobre et al. 2000; Torra et al. 2000; Yamamoto et al. 2005). While detailed mechanisms underlying clock resetting by GR have not been fully defined, GR appears to be a key entrainment signal of peripheral clock rhythms, allowing for the coupling of food- and SCN-derived resetting cues.

4.5 Circadian Control of Metabolic Peptide Hormones

Several metabolic hormones also display circadian oscillations and likely integrate circadian rhythms with feeding responses (reviewed in Kalsbeek and Fliers 2013). Diurnal oscillations of leptin and ghrelin, two hormones produced by the adipocytes and stomach, respectively, are important for delivering nutritional cues to the brain and establishing feeding behavior (Ahima et al. 1998; Huang et al. 2011; Kalra et al. 2003; Kalsbeek et al. 2001; Mistlberger 2011; Yildiz et al. 2004). Leptin levels in humans peak at night and are responsible for nocturnal appetite suppression mediated by hypothalamic neurons (Sinha et al. 1996). Conversely, ghrelin increases before meal times and facilitates food anticipatory behavior (Cummings et al. 2001). Although the mechanisms controlling the rhythmic release of leptin and ghrelin from peripheral tissues remain unclear, it is known that their effects modulate the hypothalamic clock. Thus, these hormones counterbalance the GC/GR pathway, providing information from peripheral food-responsive clocks to the central pacemaker within the SCN.

Recent studies have also uncovered a role for the pancreatic β-cell clock in normal islet insulin exocytosis (Marcheva et al. 2010, 2011). As insulin is a primary regulator of blood glucose levels, these findings highlight the critical importance of the circadian system for the maintenance of energy homeostasis. Clock-dependent insulin levels may also have a broad impact on various nutrient-sensing pathways such as signaling through AMPK or sirtuins in other tissues. Interestingly, insulin, which is able to reach the brain via a transporter across the blood–brain barrier, is in turn able to modulate circadian feeding behavior (Gerozissis 2003). Thus, this feedback loop adds another layer to the intricate interplay between the circadian and metabolic systems that ultimately increases the organism's adaptability and chances for survival.

5 Perspectives on Timing in Animal and Human Studies

5.1 Importance of Timing and Environmental Light in Experimental Design

As compelling evidence for the vast effect of the biological clock on metabolic processes continues to mount, the importance of understanding the effect of circadian rhythmicity in the treatment and design of animal and clinical studies is becoming increasingly clear. Since a vast number of metabolic genes display diurnal tissue-specific variations in expression, comprehensive analysis of metabolic processes and pathways in animals should involve studies at several different time points over a 24-h period (Lamia et al. 2008; Marcheva et al. 2010; Sadacca et al. 2010). Phase delay between the timing of transcription and translation should also be considered, since processes such as pre-mRNA splicing, polyadenylation, and RNA decay may influence the activity and function of enzymes involved in metabolic homeostasis (reviewed in Staiger and Koster 2011; O'Neill et al. 2013). For instance, in liver, rhythmic transcription of the mitochondrial succinate dehydrogenase (*Sdh1*) is phase-advanced compared to its translation, while the inhibitor of serine protease *Serpina1d* exhibits a nonrhythmic mRNA pattern, but an oscillating protein profile (Reddy et al. 2006). Finally, circadian regulation of posttranslational modification, such as protein phosphorylation and ubiquitinylation, may also affect metabolic function (Eide et al. 2005; Lamia et al. 2009; Lee et al. 2001, 2008).

Temporal factors in the environment can also influence reproducibility of research results. Light is a powerful synchronizer of circadian rhythms, and thus, animal facility lighting intensity and photoperiod can affect behavioral activity and metabolic homeostasis (Menaker 1976; Reiter 1991). For example, exposure to constant light affects catecholamine, ACTH, and progesterone levels, while constant darkness shifts the peaks of blood glucose and nonesterified fatty acids and alters the expression of catabolic enzymes (Ivanisevic-Milovanovic et al. 1995; Zhang et al. 2006). Even small changes to the normal light/dark cycles, such as a switch to daylight savings time or a brief light pulse during the dark phase, can cause temporary misalignment of behavioral and metabolic rhythmicity, potentially increasing variability in experimental results (Clough 1982). Further, contamination of light at night in animal facilities, from translucent observation door windows, poorly insulated doorframes, and in-room lighted equipment, should be minimized, as studies have reported that as little as 0.2 lux light exposure during the dark cycle can disrupt the circadian rhythms of gene expression, shift the timing of food consumption, increase body mass, reduce glucose tolerance, alter melatonin rhythms, and increase oncogenicity (Dauchy et al. 1999; Fonken et al. 2010; Minneman et al. 1974). These observations underscore the importance of taking circadian timing and environmental light cycles into consideration in the design and interpretation of metabolic studies.

5.2 *Clinical Aspects of Timing*

As scientists continue to uncover links between the circadian network and metabolism at the molecular level, many of these findings have made their way into the clinical realm in both the diagnosis and treatment of various metabolic disorders. For example, cortisol, an adrenal hormone essential for lipid and glucose metabolism, and ACTH, the pituitary hormone that regulates cortisol secretion from the adrenal gland, exhibit robust circadian rhythms in man (Orth et al. 1979; Orth and Island 1969; Szafarczyk et al. 1979). Therefore, proper diagnosis of Cushing's disease (characterized by cortisol excess) necessitates measurement of saliva cortisol in the late evening hours when the levels of this hormone are typically low, while adrenal insufficiency is more appropriately diagnosed when cortisol is measured in the morning hours when it is at its peak. Further, glucocorticoid therapy for patients with adrenal insufficiency aims to mimic the endogenous rhythms of cortisol, as short-acting synthetic glucocorticoids are usually given 2–3 times a day in tapering doses such that the largest amount is taken in the morning and the smallest in the early evening (Arlt 2009).

Melatonin, a natural hormone important for the initiation and maintenance of sleep, is another example where timing of drug delivery is critical in the treatment of daytime sleepiness following shift work or jetlag. Physiologic doses of melatonin during the day (when melatonin is normally low) result in daytime sleepiness, while treatment during the dark phase (coinciding with the endogenous increase in melatonin secretion) improves sleep latency and helps achieve continuous sleep (Brzezinski et al. 2005). Further, melatonin administration several hours prior to the normal onset of secretion causes a phase advance in the endogenous melatonin rhythm, which is particularly useful for treatment of eastbound jetlag. Conversely, melatonin treatment following the endogenous onset of secretion is often useful to improve westbound jetlag (Herxheimer and Petrie 2002).

As our knowledge of the complexity of the circadian and metabolic interacting networks deepens, we can also begin to rationally develop new treatments for disorders affected by circadian misalignment. For example, unbiased drug discovery screens have identified several compounds that can shift the phase of the endogenous clock. Indeed, treatment of human U20S cells stably expressing a *Bmal1-Luc* reporter construct with more than 120,000 potential drugs uncovered numerous compounds that either shorten or lengthen the period, including various inhibitors of CKIδ, CKIε, and GSK-3 (Hirota and Kay 2009; Hirota et al. 2008). This novel approach provides a means to pharmacologically control the circadian cycle, which may be useful in the treatment of circadian disorders and metabolic disturbances with a circadian component (reviewed in Antoch and Kondratov 2013). It also offers new insight into the interaction of previously unsuspected pathways with the circadian system.

6 Conclusion

The circadian clock is an evolutionarily conserved internal timekeeping mechanism that synchronizes endogenous systems with daily environmental cycles. The clock network is present in almost all mammalian tissues and governs a remarkable variety of biochemical, physiological, and behavioral processes. A growing body of evidence indicates that proper function of central and peripheral clocks is crucial for the well-being of the organism. Disruption of circadian rhythmicity has been implicated in the pathogenesis of several diseases, including metabolic disorders. Therefore, a deeper understanding of the role of the molecular clock in regulation of daily physiological processes will enable development of new treatments, more efficient therapeutic delivery, and better preventative strategies for management of diabetes, obesity, and other metabolic disorders.

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The Circadian Control of Sleep

Simon P. Fisher, Russell G. Foster, and Stuart N. Peirson

Abstract The sleep/wake cycle is arguably the most familiar output of the circadian system, however, sleep is a complex biological process that arises from multiple brain regions and neurotransmitters, which is regulated by numerous physiological and environmental factors. These include a circadian drive for wakefulness as well as an increase in the requirement for sleep with prolonged waking (the sleep homeostat). In this chapter, we describe the regulation of sleep, with a particular emphasis on the contribution of the circadian system. Since their identification, the role of clock genes in the regulation of sleep has attracted considerable interest, and here, we provide an overview of the interplay between specific elements of the molecular clock with the sleep regulatory system. Finally, we summarise the role of the light environment, melatonin and social cues in the modulation of sleep, with a focus on the role of melanopsin ganglion cells.

Keywords Sleep • Circadian • Clock gene • Melatonin • Melanopsin

1 Introduction

The regular cycle of sleep and wakefulness is perhaps the most obvious 24-h oscillation. However, sleep is a complex physiological process involving the interaction of multiple neurotransmitter systems and a diverse network of mutually inhibiting arousal and sleep-promoting neurons. This highly coordinated neural

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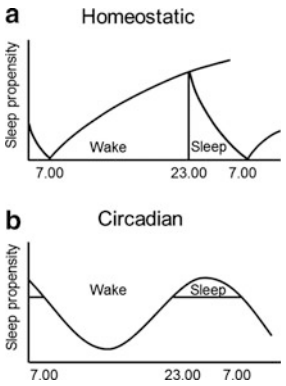

Fig. 1 Sleep regulation by homeostatic and circadian mechanisms. (**a**) The homeostatic drive for sleep increases sleep propensity with prolonged wakefulness. Sleep pressure declines following sleep, but increases again at waking. (**b**) Circadian drive. The circadian regulation of sleep creates a drive for wakefulness during the day, which declines at night. As such, sleep propensity is low during the day, but increases at night. Figure based on that of Borbely (1982)

activity drives alternating patterns of behaviour characterised by changes in rest/activity, body posture and responsiveness to stimuli (Tobler 1995). Reflecting the complexity of the neurobiological processes involved, sleep is regulated by a range of internal and external drivers. In this chapter, we will discuss these parameters, with a particular focus on the contribution of the circadian clock and its interaction with the sleep/wake regulatory system.

The primary measures used to define sleep in mammals are the electroencephalogram (EEG) and electromyogram (EMG) which are used to characterise sleep as either rapid eye movement (REM) or non-rapid eye movement (NREM) states. This gold standard approach of classifying sleep not only enables the assessment of sleep structure but also permits power spectral analysis of the EEG for different sleep/wake states. In 1982, Borbely proposed the 'two process model' of sleep regulation which provides a conceptual framework for understanding the timing and structure of sleep/wake behaviour. It describes a homeostatic process (S), which increases as a function of the duration of wakefulness and a circadian process (C), determining the timing of sleep and wakefulness (Borbely 1982) (Fig. 1). In humans the consolidation of wakefulness into a single bout is a result of the phase relationship between these two processes where the circadian drive for arousal opposes the increasing propensity to sleep across the day (Dijk and Czeisler 1995). There has been considerable progress in our understanding of the circadian process with the anatomical location of the master circadian pacemaker identified within the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. However, relatively less is known regarding the molecular and cellular processes underlying sleep homeostasis and its interaction with the circadian timing system.

2 Homeostatic Regulation of Sleep

The homeostatic process regulates the propensity for sleep, which increases exponentially at the onset of wakefulness and subsequently diminishes during sleep (Fig. 1a). It is functionally distinct from the circadian system since rodents with lesions of the SCN continue to exhibit a strong compensatory increase in sleep after total sleep deprivation (Mistlberger et al. 1983; Tobler et al. 1983). The best characterised marker of sleep homeostasis and a correlate of sleep intensity is EEG slow-wave activity (SWA, 0.5–4 Hz) during NREM sleep which increases as a function of the duration of prior wakefulness and declines exponentially across a typical sleep episode (Borbely et al. 1981; Lancel et al. 1991). It has been suggested that this homeostatic decrease in SWA during sleep is associated with a downscaling of synaptic strength and is important for the positive effects of sleep on neural function (Tononi and Cirelli 2006). EEG power in the theta frequency range (5–7 Hz) has also been identified to reflect sleep propensity during quiet wakefulness. Notably studies in both rodents and humans have demonstrated that a rise in EEG theta power during enforced wakefulness was able to predict the increase in EEG SWA during subsequent sleep (Finelli et al. 2000; Vyazovskiy and Tobler 2005). It is now appreciated that EEG SWA is topographically represented in the cortex with changes in SWA occurring in restricted brain regions with different temporal dynamics (Rusterholz and Achermann 2011; Zavada et al. 2009) which have been associated with alterations in learning and performance (Huber et al. 2004, 2006; Murphy et al. 2011). Importantly this provides evidence that the regulation of SWA and sleep homeostasis can occur at a local level. A recent study has further reinforced this concept of 'local' sleep regulation after identifying discrete cortical regions of the rat brain that effectively go 'offline' during a period of prolonged wakefulness even though the animal remains awake by the assessment of global EEG parameters (Vyazovskiy et al. 2011).

Identifying a neuroanatomical basis of homeostatic sleep regulation has been extremely challenging and remains one of the outstanding questions in sleep research. Sleep-promoting neurons have been previously identified in the ventrolateral preoptic area (VLPO) and median preoptic nucleus (MnPO) of the hypothalamus (Gong et al. 2004; Sherin et al. 1996); however, the recent discovery of a population of sleep-active neurons in the cortex has further supported an anatomical basis of homeostatic sleep regulation (Gerashchenko et al. 2008; Pasumarthi et al. 2010). These sleep-active cells expressing neuronal nitric oxide synthase are a subpopulation of GABAergic interneurons that project long distances throughout the cerebral cortex with the number of cells activated during sleep proportional to SWA intensity (Gerashchenko et al. 2008). Determining the neuronal circuitry and mechanisms that result in the activation of these cells during sleep will further increase our understanding of their potential role in homeostatic sleep regulation.

Research has also focused on the role of chemical mediators in the regulation of sleep homeostasis. Such a mediator would be expected to accumulate after prolonged wakefulness or sleep deprivation and decline during sleep. Several

candidate substances have been proposed but particular focus has been placed on the purine nucleoside adenosine (Basheer et al. 2004). Microdialysis studies in cats have demonstrated that adenosine selectively increases in the basal forebrain (BF) during 6 h of sleep deprivation (Porkka-Heiskanen et al. 1997, 2000). Furthermore, perfusion of adenosine into the BF of freely moving cats reduces wakefulness, decreases cortical arousal (Portas et al. 1997) and activates neurons in the VLPO (Scammell et al. 2001). A study employing a significantly longer period of sleep deprivation (11 h) has shown that initially nitric oxide followed by adenosine accumulates in the BF, with levels of these molecules increasing in the frontal cortex several hours later, providing further insight into the temporal dynamics of sleep homeostasis (Kalinchuk et al. 2011). Caffeine, a potent stimulant, functions as an adenosine antagonist at both A_1 and A_2 receptors. Studies involving knockout mice for these receptors indicate that blockade of the A_2 receptor mediates these wake-promoting effects since caffeine could promote arousal in wild-type and A_1 knockout mice but not in mice deficient in the A_{2A} receptor (Huang et al. 2005). In addition, caffeine administered to young male subjects during sleep deprivation reduced subjective sleepiness and EEG theta activity and decreased SWA during subsequent recovery sleep (Landolt 2004). This ability of caffeine to reduce the accumulation of sleep propensity after prolonged wakefulness further proposes a critical role of adenosine in sleep homeostasis. Prostaglandin D_2 (PGD_2), has also been identified as a putative endogenous sleep-promoting factor (Huang et al. 2007), and evidence suggests it may also mediate its effects on sleep through A_{2A} receptors (Satoh et al. 1996).

3 Circadian Regulation of Sleep

The circadian influence on sleep was soon appreciated after it was demonstrated that rhythms of sleep and wakefulness persist in free-running conditions and are strongly synchronised to the rhythm of core body temperature (Czeisler et al. 1980). The circadian regulation of sleep is independent of prior wakefulness and determines the phases of high and low sleep propensity across the 24-h day (Fig. 1b; Borbely and Achermann 1999). Studies in humans using forced desynchrony protocols (enforced 28-h sleep/wake cycles) have revealed the uncoupling of the sleep/wake cycle from endogenous circadian processes and further support the dualistic view of the control of sleep (Dijk and Lockley 2002). Human volunteers scheduled to a 28-h rest/activity cycle were only able to sleep undisturbed for an 8-h period when sleep initiation occurred 6 h before the endogenous circadian temperature minimum (Dijk and Czeisler 1994). Evidence strongly suggests that the circadian process arises from the SCN located in the anterior hypothalamus (Weaver 1998). In rodents, targeted lesioning of the SCN disrupts circadian rhythms in locomotor activity, feeding and drinking (Stephan and Zucker 1972; Moore 1983), whilst surgically implanted SCN tissue grafts can restore rhythms with a period determined by the donor, not the recipient (Ralph et al. 1990; King et al. 2003).

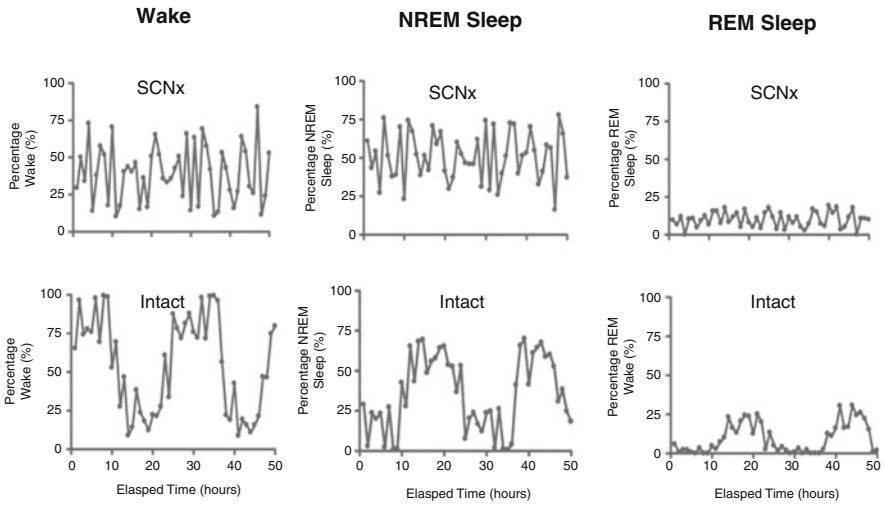

Fig. 2 Circadian rhythms in wakefulness, NREM and REM sleep are abolished in SCN-lesioned rats. Wakefulness, NREM and REM sleep plotted for 50 consecutive hours in constant darkness for an individual SCN-lesioned rat (SCNx, *top panels*) and a rat with an intact SCN (*bottom panels*). Data points represent hourly percentage values for an individual rat (Figure based on unpublished data, S. Fisher)

These transplantation studies were essential in confirming the SCN as the principal mammalian timekeeping structure. Through a number of intermediate relay nuclei, the SCN innervates multiple brain areas involved in sleep/wake cycle regulation including the VLPO and the MnPO areas (Deurveilher and Semba 2005). The SCN is known to receive information relating to the specific sleep/wake states, as SCN electrical firing is modified by changes in vigilance state in the rat (Deboer et al. 2003). Neuronal activity in the SCN was lowered during NREM sleep and by contrast was increased when the rat entered periods of REM sleep independent of circadian phase (Deboer et al. 2003). Furthermore, after prolonged sleep deprivation (6 h), the circadian amplitude of SCN electrical activity was reported to be suppressed during recovery sleep, an effect persisting for up to 7 h (Deboer et al. 2007). This suggests that sleep deprivation directly modulates the electrical rhythm of the circadian clock in addition to its well-characterised effects on the sleep homeostat.

Lesions of the SCN in rodents also leads to a disruption and flattening of the rhythm of sleep and wakefulness, where animals no longer display consolidated episodes of NREM and REM sleep and instead exhibit numerous transitions between states together with frequent arousals (Fig. 2). The ‘opponent process’ model proposed by Edgar and colleagues specifies that the circadian process actively promotes the initiation and maintenance of wakefulness opposing the homeostatic drive for sleep (Edgar et al. 1993). This hypothesis is primarily based on SCN lesion studies performed in squirrel monkeys which results in an increase in total sleep time compared to sham-operated controls (Edgar et al. 1993).

Similar observations have been made in mice where SCN lesions increase sleep time by ~8.1 % (Easton et al. 2004), suggesting a wider role for the SCN in sleep regulation beyond purely the timing of vigilance states. By contrast, the majority of SCN lesion studies performed in rats do not result in major changes in the total amount of sleep (Eastman et al. 1984; Mistlberger et al. 1987; Mouret et al. 1978), and homeostatic regulation of sleep is also preserved in arrhythmic hamsters (Larkin et al. 2004). This apparent controversy may suggest that the SCN has both a wake- and sleep-promoting action, promoting arousal at one time of the day and sleep at a different time, possibly by changing the balance of its output signal (Dijk and Duffy 1999; Mistlberger et al. 1983).

3.1 Clock Genes and Sleep

The important role that clock genes play in the generation of circadian rhythms is well established. Over the last 20 years, there has been remarkable progress in our understanding of the molecular mechanisms responsible for generating the cell-autonomous oscillations which make up the circadian system. This autoregulatory network relies on the interaction between both positive and negative transcriptional/translational feedback loops. In mammals, the transcription factors CLOCK and BMAL1 form a heterodimeric complex which drives the transcription of the *Period* (*Per 1*, *2*, *3*) and *Cryptochrome* (*Cry1*, *2*) genes through binding to E-box promoter sequences (CACGTG) (Gekakis et al. 1998). Additionally, the transcription factor neuronal PAS domain protein 2 (NPAS2), an analogue of CLOCK, is expressed in the forebrain nuclei, basal ganglia and limbic system (Garcia et al. 2000). NPAS2 can also heterodimerise with BMAL1 to activate the transcription of *Per* and *Cry* genes (Reick et al. 2001). Whilst it was originally suggested that NPAS2 is not found in the SCN (Garcia et al. 2000), later studies showed that it is both expressed in the SCN and can functionally substitute for CLOCK (DeBruyne et al. 2007). PER and CRY proteins are synthesised in the cytoplasm and form complexes that are phosphorylated by casein kinases I δ and ε which subsequently re-enter the nucleus and bind to CLOCK/NPAS2:BMAL1 heterodimers to inhibit their own transcription (Reppert and Weaver 2002). Formation of CLOCK/BMAL1 heterodimers can also result in the activation of the retinoic acid-related orphan nuclear receptors Rora and Rev-erbα. Rev-erbα can inhibit CLOCK and BMAL1 expression, whilst by contrast, RORA is an activator which functions to reinforce oscillations and increase levels of *Bmal1* in the absence of PER and CRY proteins (Buhr and Takahashi 2013; O'Neill et al. 2013). In the mammalian circadian clock, a certain degree of overlap exists as single mutations in the clock genes *Per* and *Cry* do not result in arrhythmicity (Bae et al. 2001; Okamura et al. 1999). Additionally, in mice *Per3* is not required for circadian rhythm generation with only minor effects on circadian period reported in its absence (Shearman et al. 2000).

Studies involving clock gene mutant mice have allowed a finer dissection of the role of individual clock components in circadian rhythm generation but have also

shed new light on their potential role in the regulation of sleep. The advantage of using genetic approaches to disrupt the circadian system are that SCN neuronal connections remain largely intact; however, developmental effects of the gene knockout cannot be excluded. Below, we summarise the role of specific clock genes and clock-controlled genes in the regulation of sleep, including their effects on the total amount of sleep, sleep structure and the EEG. These data are summarised in Table 1.

3.1.1 *Cryptochrome*

Cry1 and *Cry2* double-knockout mice ($Cry1,2^{-/-}$) are rhythmic under a regular light/dark cycle but arrhythmic under constant conditions (van der Horst et al. 1999; Vitaterna et al. 1999). Cyclic expression of the *Per* genes is eliminated in both the SCN and peripheral tissues in these mice (Okamura et al. 1999), although they display normal masking responses to light (Mrosovsky 2001). In addition, $Cry1,2^{-/-}$ mice generated on the C3H melatonin-proficient background fail to show a circadian rhythm in melatonin production, one of the most reliable output measures of the circadian clock (Yamanaka et al. 2010). However, independent of whether they display rhythmicity, $Cry1,2^{-/-}$ mice exhibit a 1.8-h increase in NREM sleep with an approximate 40 % increase in NREM sleep bout duration (Wisor et al. 2002). Furthermore, they show an elevation of EEG SWA during baseline recordings and after sleep deprivation, indicating that the absence of both *Cry* genes leads to increases in the accumulation of sleep pressure. $Cry1,2^{-/-}$ mice also fail to show the typical compensatory rebound in NREM sleep after enforced wakefulness (Wisor et al. 2002). This sleep phenotype cannot be ascribed to either of the *Cry* genes alone since it is not replicated in single *Cry* knockout mice (Wisor et al. 2008). The $Cry1,2^{-/-}$ mouse phenotype appears to be more complex than simply a genetic model of arrhythmia and actually implicates a larger role for *Cry* genes in the homeostatic sleep regulation.

3.1.2 *Period*

Mice with mutations in both *Per1* and *Per2* ($Per1,2^{-/-}$) genes exhibit robust diurnal rhythms only under a standard light/dark cycle. In contrast to $Cry1,2^{-/-}$ mice, they show no change in the total amount of sleep across a 24-h period under a regular L:D cycle (Kopp et al. 2002) or under constant darkness (Shiromani et al. 2004). Similarly EEG recordings performed in single mutant *Per1* and *Per2* mice did not find any alteration in total sleep time and demonstrated that they have a normal homeostatic response to sleep deprivation (Kopp et al. 2002). After sleep deprivation, $Per1,2^{-/-}$ mice exhibit the expected increase in EEG SWA in NREM sleep, suggesting the homeostatic control of sleep is intact. A more recent study using Per3 knockout mice ($Per3^{-/-}$) on the C57BL/6J background identified differences in the temporal distribution of sleep with an increase in NREM and REM sleep immediately after the dark/light transition (Hasan et al. 2011). This is

Table 1 Summary of sleep phenotype of clock gene mutant/knockout models

	Total sleep (24 h)						
Gene	LD	DD	Baseline EEG	REM/NREM	Sleep deprivation	Other effects	References
Cry1/2$^{-/-}$	1.8 h ↑ in NREM	1.5 h ↑ in NREM	NREM delta power ↑	Attenuated sleep/wake rhythm across LD cycle	Compensatory response to sleep loss ↓	NREM bout duration ↑	Wisor et al. (2002)
Per1/2$^{ldc/ldc}$	No effect	No effect	No effect on NREM delta or REM theta power	Wake time in L ↑	Compensatory response to sleep loss	Longer wake bouts in D Temperature in D period ↓	Shiromani et al. (2004)
Bmal1$^{-/-}$	1.5 h ↑ in total sleep	6.2 % ↑ in NREM	NREM delta power ↑ Flattened distribution of EEG delta power	Arrhythmic sleep/wake states in DD	REM sleep rebound ↓	↑ body temperature at L–D transition absent Sleep fragmentation ↑	Laposky et al. (2005)
Clock^{m1Jt}	2 h ↓ in total sleep	1 h to 2 h ↓ in NREM	Total NREM delta energy in 24-h baseline ↓ No effect on NREM delta power No effect on in EEG theta or sigma power	NREM sleep in L ↓	Compensatory response to sleep loss REM sleep rebound ↓	NREM/REM sleep onset latency ↓ NREM bout duration ↓ Obesity, metabolic syndrome, diabetes	Naylor et al. (2000) Turek et al. (2005) Marcheva et al. (2010)
Npas2$^{-/-}$	~40 min ↓ in NREM		Activity in spindle frequency ↓ Shift of NREM delta power to faster frequency	Wake time in D phase ↑ NREM/REM in D phase ↓	Compensatory response to sleep loss ↓ (*male mice only*)		Dudley et al. (2003), Franken et al. (2006)
Dec2^{P385R}			No effect on NREM delta or REM theta power	NREM/REM in L phase ↓	Compensatory response to sleep loss ↓	NREM episodes in L ↑ Sleep fragmentation ↑	He et al. (2009)

PK2$^{-/-}$	1.3 h ↓ total sleep	1.3 h ↓ total sleep	EEG theta power ↓ in REM	REM sleep duration ↑	Compensatory response to sleep loss ↓	NREM/REM sleep onset latency ↓ Less responsive to environmental arousal	Hu et al. (2007)
Dbp$^{-/-}$	No effect	No effect	Amplitude of delta power ↓ NREM delta power ↓ in D period Theta frequency peak in REM sleep ↑	Sleep during L ↓ Sleep during D ↑ Circadian amplitude of the sleep distribution ↓	Compensatory response to sleep loss REM sleep rebound absent	Greater disruption in DD than under LD and ↓ sleep amplitude	Franken et al. (2000)
Vipr2$^{-/-}$	~50 min ↑ in NREM	No effect	Normal EEG delta power in NREM sleep	Less defined sleep and wake phases in D and L		Sleep/wake transitions and brief arousals ↑ Ultradian cycles of sleep/wake in DD	Sheward et al. (2010)
Per3	No effect	No effect	Temporal distribution of sleep altered EEG delta power in D period ↑ Theta frequency peak in REM sleep ↓	NREM/REM after D–L transition ↑	Accumulation of EEG delta power ↑ in recovery sleep Number of NREM sleep bouts ↑	Running-wheel activity ↑ in D	Shiromani et al. (2004) Hasan et al. (2011)

L stands for Light
D stands for Dark

suggestive of an enhanced response to the sleep-promoting effects of light; however, this appears to be in contrast to the effects of light on running-wheel activity in *Per3*$^{-/-}$ mice, where a reduction in negative masking and a shorter free-running period under constant light were reported (van der Veen and Archer 2010). *Per3*$^{-/-}$ mice show enhanced accumulation of EEG delta power across the active period (Hasan et al. 2011). This increased sleep pressure in *Per3*$^{-/-}$ mice may explain the increase in sleep observed early in the light period. *Per* gene expression can also be modulated through manipulations of homeostatic sleep pressure with an elevation of *Per1* and *Per2* in the cortex detected after sleep deprivation (Wisor et al. 2002). Additionally, studies in humans have linked functional polymorphisms in the *Per3* gene to differences in sleep homeostasis in terms of EEG SWA in NREM sleep but also in theta and alpha frequencies during wakefulness and REM sleep (Viola et al. 2007). A polymorphism in the promoter region of *Per3* has been recently associated with delayed sleep-phase syndrome, a situation where sleep/wake timing of the individual is delayed relative to the external light/dark cycle (Archer et al. 2010). Overall evidence suggests that unlike the *Cry* genes, *Per1* and *Per2* are not implicated in homeostatic sleep regulation, but indicate an emerging role for *Per3* in sleep homeostasis.

3.1.3 *Bmal1*

Both *Cry* and *Per* gene expression are under the control of CLOCK/NPAS2 and BMAL1 heterodimers. Mutations in these genes indicate they are important in regulating circadian function but interestingly also impact the underlying sleep phenotype. *Bmal1*$^{-/-}$ mice are arrhythmic and exhibit decreased activity levels when kept under a regular light/dark cycle or constant conditions (Bunger et al. 2000). These mice exhibit a 1.5-h increase in total sleep predominantly due to an increase in NREM and REM sleep during the active phase (Laposky et al. 2005). *Bmal1*$^{-/-}$ mice also failed to show the predictable increase in arousal or body temperature during the light/dark transition, indicating potential defects in light input pathways to the SCN. Sleep was highly fragmented in these animals with an increase in the number of sleep bouts during the light period. They also lacked a rhythm in sleep propensity, as indicated by the flattened distribution of EEG delta power in NREM sleep, which was also elevated under baseline conditions indicating they function under a high level of sleep pressure. However, the REM sleep rebound after sleep deprivation was attenuated in *Bmal1*$^{-/-}$ mice. This modulation of both sleep amount and intensity in *Bmal1* mutant mice suggests a role for this clock gene in the homeostatic regulation of sleep.

3.1.4 *Clock*

A mutagenesis screen performed by Joseph Takahashi and colleagues led to the discovery of *Clock*, the first mammalian gene identified to be important for normal circadian function (Vitaterna et al. 1994). A dominant negative mutation of this

gene resulted in a lengthening of circadian period and arrhythmicity in homozygous *Clock* mutants under free-running conditions but not under a regular L:D cycle (Vitaterna et al. 1994). In mice heterozygous and homozygous for the *Clock* mutation, total sleep time was decreased by 1 and 2 h, respectively, compared to wild-type animals (Naylor et al. 2000). NREM sleep bout duration was also significantly reduced in *Clock* homozygous mice, although EEG delta power in NREM sleep remained unaffected, indicating that the decrease in the length of sleep was not compensated by changes in sleep intensity (Naylor et al. 2000). Additionally, these animals showed a normal rebound in sleep after 6 h sleep deprivation. This infers the *Clock* gene is important in regulating sleep amount and timing but is not critical for the functioning of all aspects of homeostatic sleep regulation. Furthermore, it should be noted that *Clock*-mutant animals display a complex phenotype, including obesity, metabolic syndrome (Turek et al. 2005) and diabetes (Marcheva et al. 2010) which may also impact changes in the sleep/wake cycle. The central role of CLOCK in the circadian oscillator was challenged when it was shown that in contrast to *Clock* mutants, $Clock^{-/-}$ mice show robust circadian rhythms of locomotor activity (Debruyne et al. 2006). However, this may be explained by the functional substitution of NPAS2 compensating in *Clock* knockouts (see below).

3.1.5 Other Canonical Clock Genes

NPAS2, an analogue of *Clock*, is a basic helix–loop–helix PAS domain transcription factor. It forms a heterodimeric complex with BMAL1 leading to the transcription of the negative regulators *Cry* and *Per*. NPAS2 is expressed in the forebrain nuclei, basal ganglia and limbic system (Garcia et al. 2000), as well as the SCN where it can substitute for CLOCK (DeBruyne et al. 2007). This substitution of NPAS2 for CLOCK is likely to account for the difference in phenotype between *Clock*-mutant and knockout mice (Debruyne et al. 2006). Wheel-running studies performed in $Npas2^{-/-}$ mice demonstrate a reduction in the free-running period, increases in the rates of re-entrainment and attenuation of the typical 'rest phase' in the second half of the dark period (Dudley et al. 2003). The authors confirmed the latter observation using EEG recordings demonstrating that $Npas2^{-/-}$ mice remained awake for a greater proportion of the dark period with reductions in NREM and REM sleep. These mice also display a reduction in the amount of recovery sleep following sleep deprivation, a difference that was only apparent in male $Npas2^{-/-}$ mice (Franken et al. 2006). These mice also show changes in the EEG during NREM sleep, with a reduction in activity in the spindle frequency range (10–15 Hz) and a shift of delta activity towards faster frequencies, signifying a role for NPAS2 in the propagation of EEG oscillations (Franken et al. 2006). To date, no study has investigated sleep in $Clock^{-/-}$ or *Clock/Npas2* double-knockout mice.

The basic helix–loop–helix transcription factors *Dec1* (Sharp2) and *Dec2* (Sharp1) are expressed in a circadian manner in the SCN and are important regulatory components of the molecular clock. They act primarily as negative

modulators which repress CLOCK/BMAL-induced gene expression of the Per1 promoter (Honma et al. 2002). Studies in mice deficient in *Dec1* and *Dec2* indicate a role for these transcription factors in the control of period length, phase resetting and circadian entrainment (Rossner et al. 2008). A point mutation in *Dec2* has been associated with a short sleep phenotype in humans (He et al. 2009). The small sample size in this study led the investigators to test this linkage by replicating the $DEC2^{P385R}$ mutation in a murine model. They were convincingly able to reproduce this short sleep phenotype in mice that exhibited decreases in NREM and REM sleep in the light phase and an increase in sleep fragmentation (He et al. 2009). Furthermore, the DEC2 mutation led to a decrease in NREM sleep following sleep deprivation and a reduction in EEG delta power confirming the involvement of this clock component in homeostatic sleep regulation. By contrast, only minimal changes in sleep were observed in *Dec2* knockout mice, although the compensatory rebound of NREM sleep after sleep deprivation exhibited considerably slower kinetics, suggesting a role for *Dec2* in the fine-tuning of sleep regulation (He et al. 2009).

3.2 Clock Gene Expression After Sleep Deprivation

During sleep, a number of genes are upregulated in the brain, and microarray analysis demonstrates that ~10 % of the transcripts in the cerebral cortex alter their expression between day and night (Cirelli et al. 2004). Many of the 1,500 genes that change expression across the 24-h day are linked to changes in behavioural state rather than to time of day differences. Surprisingly, after sleep deprivation, very few genes alter their expression; these are typically genes involved in neuronal protection and recovery (Maret et al. 2007). Sleep deprivation can also modify the expression of clock genes in areas outside the SCN. *Per* levels are elevated when sleep drive is high, which occurs independently of circadian phase (Abe et al. 2002; Mrosovsky et al. 2001). In the forebrain, both *Per1* and *Per2* mRNA levels increase after sleep deprivation in mice (Wisor et al. 2008) with a decrease in the clock-controlled gene *Dbp* (Franken et al. 2007). Clock gene expression has also been characterised in inbred strains of mice, which present differences in sleep rebound after enforced wakefulness. These studies have identified a relationship between the expression of *Per1* and *Per2* with the length of time spent awake and are consistent with a role for these clock genes in homeostatic sleep regulation (Franken et al. 2007). At the level of the EEG, changes in clock gene expression after sleep deprivation were also found to be proportional to the increase in EEG delta power across different strains of mice (Wisor et al. 2008). A potential mechanism by which sleep deprivation could alter clock gene expression has recently been described. DNA binding of CLOCK and BMAL1 to target clock genes varies over the circadian cycle in the cerebral cortex, peaking around ZT 6. Sleep deprivation was shown to reduce CLOCK and BMAL1 activation of *Dbp* and *Per2*, but not *Per1* and *Cry1*. As such, sleep history may directly regulate the circadian clock in tissues outside the SCN (Mongrain et al. 2011).

3.3 Clock-Related Genes

In addition to the core clock machinery, there are a number of clock-controlled genes which have been shown to be involved in the regulation of sleep. Expression of prokineticin 2 (*Prok2*), a putative clock-controlled output signal, is thought to be important in the transmission of circadian rhythms. *Prok2*$^{-/-}$ mice exhibit a reduction in the circadian amplitude of activity, core body temperature and sleep/wake cycle (Li et al. 2006). *Prok2*$^{-/-}$ mice sleep ~1 h 30 min less than wild-type mice over a 24-h period, changes that remained apparent under constant darkness indicating that they were not due to a masking effect of light. Remarkably, deficiency of the *Prok2*$^{-/-}$ gene modifies NREM and REM sleep in opposing directions. A reduction in NREM sleep was observed during the light period, whilst increased REM sleep occurred during both light and dark phases despite an overall reduction in total sleep (Hu et al. 2007). In these mice, NREM and REM sleep latencies were also shorter in *Prok2*$^{-/-}$ mice, indicating higher sleep pressure (Hu et al. 2007). EEG SWA during NREM sleep was comparable in *Prok2*$^{-/-}$ and wild-type mice; however, EEG theta power in REM sleep was decreased in *Prok2*$^{-/-}$ mice which also exhibited attenuated compensatory responses to sleep deprivation. These studies highlight a role for *Prok2* in the regulation of the circadian process but also in sleep homeostasis, further indicating the large degree of crosstalk between these two major processes governing the regulation of sleep.

The PAR leucine zipper transcription factor *Dbp* is under transcriptional control of CLOCK (Ripperger et al. 2000). *Dbp*$^{-/-}$ mice display a mild circadian phenotype remaining rhythmic but exhibiting a shorter circadian period (approximately 30 min) and a reduction in activity levels (Lopez-Molina et al. 1997). Total sleep duration was not altered in *Dbp*$^{-/-}$ mice, but the circadian amplitudes of sleep time and sleep consolidation were reduced, suggesting that *Dbp* may be important in altering the magnitude of the output signal from the circadian clock. REM sleep was reduced during the light period, and an increase in EEG theta frequency occurred during exploratory behaviour and in REM sleep. A normal homeostatic response to sleep deprivation was present in the absence of *Dbp*, but the accumulation of EEG delta power in the active period was reduced (Franken et al. 2000). This decrease in EEG delta power could be attributed to the slight increase in NREM sleep throughout the dark period, indicating little direct effects of *Dbp* on homeostatic sleep regulation.

Vasoactive intestinal polypeptide (VIP) signalling through the activation of the VPAC2 receptor is thought to be critical in sustaining circadian rhythms in individual SCN cells but also in the synchronisation of electrical activity between these cells (Brown et al. 2007). Mice deficient in the VPAC2 receptor gene (*Vipr2*$^{-/-}$) exhibited robust activity rhythms but showed an altered diurnal sleep/wake rhythm. Additionally, more sleep/wake transitions were evident in *Vipr2*$^{-/-}$ mice whilst total NREM sleep time was increased (~50 min) without any reported differences in NREM EEG delta power compared to wild-type mice (Sheward et al. 2010).

3.4 A Complex Role for Clock Genes in Sleep Regulation

Clock genes play a fundamental role in circadian rhythm generation, and disruption of the core clock mechanism would be expected to alter the timing of sleep; however, more surprising are its effects on the homeostatic process. Studies in transgenic mice have demonstrated that many of these genes also exert a range of effects on homeostatic sleep/wake parameters. These genetic findings are consistent with the earlier SCN lesion studies and strongly suggest that, rather than a clear separation between circadian and homeostatic processes, there is a strong interaction between these mechanisms. It will be interesting to determine how the circadian regulation of sleep is in turn affected in transgenics in which only homeostatic sleep is disturbed. In addition to their role in the core clock mechanism, it is also possible that targeted disruption of clock genes results in effects on sleep via non-circadian mechanisms. One explanation is that clock genes expressed in the SCN, responsible for the generation of circadian rhythms, are also found in other areas of the brain and cortex where they are important for regulating sleep propensity. The complexity of the findings described above, in which disruption of different clock components produces a wide range of effects on sleep, suggests that different clock genes may be involved in other molecular processes in addition to those involved in the transcriptional–translational feedback loop that generate intracellular circadian oscillations. These pleiotropic functions may directly relate to sleep or may be associated with unrelated processes such as metabolism, neurotransmission or immune functions which result in sleep disturbances (Rosenwasser 2010).

4 Regulation of Sleep by Light

The light/dark cycle provides the primary entrainment cue (zeitgeber) for the circadian system, and as a result, light will obviously modulate sleep/wake timing via photoentrainment. However, in addition to this role, acute light exposure has been shown to be involved in the regulation of sleep (Benca et al. 1998; Borbely 1978). Because of the importance of the light environment in the regulation of sleep, several groups have addressed the contribution of specific retinal photoreceptor classes in this process.

4.1 The Role of Melanopsin in Sleep Regulation

The mammalian eye serves a dual function, regulating both image-forming (IF) vision and numerous nonimage-forming (NIF) responses to light, including sleep (Lupi et al. 2008). These NIF responses to light are dependent upon retinal photoreceptors, which include the rods and cones as well as the recently identified photosensitive retinal ganglion cells (pRGCs) which express the photopigment

melanopsin (Hankins et al. 2008). Whilst many studies have focused upon the role of melanopsin in NIF responses to light, recent work by several groups has shown that rods and cones also contribute (Altimus et al. 2010; Lall et al. 2010). Mice lacking rods and cones (*rd/rd cl*) exhibit normal entrainment of sleep/wake timing and acute sleep induction in response to nocturnal light (Lupi et al. 2008). However, whilst entrainment of sleep/wake timing occurred in an attenuated form in mice lacking melanopsin photopigment ($Opn4^{-/-}$), acute sleep induction in response to a 1-h light pulse at ZT 16 was abolished. This was mirrored at a molecular level by abolition of *Fos* induction in the VLPO (Lupi et al. 2008). These findings suggested a critical role for the pRGCs in sleep regulation in response to light. In a subsequent study, Altimus et al. (Altimus et al. 2008) also reported that $Opn4^{-/-}$ mice showed no sleep induction in response to a 3-h light pulse (ZT 14–17). If, however, the data were examined in 30-min time bins, sleep did seem to occur in the first 30 min. This study also showed that mice lacking functional rods and cones ($Gnat1^{-/-}$;$Cnga3^{-/-}$) exhibited attenuated sleep induction in response to light. A mixed photoreceptor input to the sleep/arousal system was further demonstrated when mice were exposed to a 3-h dark pulse during the normal light phase (ZT 2–5) which induced wakefulness within 30 min in wild-type mice as well as in $Opn4^{-/-}$ animals and mice lacking functional rods and cones. Ablation of the melanopsin pRGCs using a transgenic model expressing an attenuated diphtheria toxin under control of the melanopsin locus ($Opn4^{DTA}$) resulted in abolition of sleep entrainment, acute sleep promotion and induction of wakefulness (Altimus et al. 2008), consistent with the hypothesis that melanopsin pRGCs form the primary conduits for irradiance detection (Guler et al. 2008). The third study by Tsai et al. (Tsai et al. 2009) found that a 1-h light pulse at ZT 15–16 failed to induce sleep in $Opn4^{-/-}$ mice, comparable with the previous two studies (Altimus et al. 2008; Lupi et al. 2008). These authors also found that a dark pulse at ZT 3–4 induced wakefulness, although this response was delayed in $Opn4^{-/-}$ animals. Additional studies using a repeated 1h:1h L:D cycle showed that the failure to demonstrate sleep induction in $Opn4^{-/-}$ mice was only apparent during the subjective night (ZT 15–21). A detailed analysis of the time course of sleep induction in response to light showed that $Opn4^{-/-}$ mice do in fact show some sleep induction in response to light but that these responses are much slower and attenuated during the dark phase (Tsai et al. 2009).

The persistence of sleep entrainment and acute sleep induction in an attenuated form in melanopsin knockout mice clearly shows that under different light stimuli, rods and/or cones are also important for sleep regulation. Whilst we now know that different photoreceptors and the quality of the light environment all contribute to sleep regulation, this relationship remains poorly defined.

5 Effects of Melatonin on Sleep

Melatonin is a neurohormone secreted by the pineal gland during the dark period of the day and has been linked with a diverse array of biological and physiological actions (Pandi-Perumal et al. 2006). The large amplitude rhythm of melatonin

represents a reliable marker of the phase of the circadian clock, transducing photoperiodic information and serving as a common humoral signal for circadian organisation (Cassone 1990; Korf et al. 1998). In addition to its chronobiotic effects, significant attention has centred on the sleep-promoting effects of melatonin and more specifically the mechanism and receptors involved. Exogenous melatonin promotes sleep in human subjects (Zhdanova 2005), although there has been controversy over its effectiveness highlighted by two contrasting meta-analyses (Brzezinski et al. 2005; Buscemi et al. 2006). The effects of pharmacological levels of melatonin on sleep in animal models present a similarly contradictory picture, with a range of studies identifying a sleep-promoting action (Akanmu et al. 2004; Holmes and Sugden 1982; Wang et al. 2003), whilst others report melatonin to be ineffective (Huber et al. 1998; Langebartels et al. 2001; Mirmiran and Pevet 1986; Tobler et al. 1994). Part of this controversy undoubtably reflects differences in dosage, time of administration and the arousal status of the subject which may preclude certain experimental protocols revealing these properties but also the subtle nature of the sleep-promoting action of melatonin.

Despite this disparity, the pharmaceutical industry has shown significant interest in exploiting the pharmacology of melatonin with a variety of melatonin agonists developed for the treatment of sleep disorders (Zlotos 2012). One of these melatoninergic compounds currently on the market is ramelteon, a non-subtype selective melatonin agonist. It exerts a sleep-promoting effect in rats (Fisher et al. 2008), mice (Miyamoto 2006), monkeys (Yukuhiro et al. 2004) and cats (Miyamoto et al. 2004). In rats, ramelteon was found to be marginally superior to melatonin in terms of the duration of action (Fisher et al. 2008), possibly reflecting its greater affinity for melatonin receptors and increased stability in vivo. Furthermore, a number of clinical studies have shown ramelteon to be effective in the treatment of both transient (Roth et al. 2005) and chronic insomnia (Liu and Wang 2012).

The mechanism responsible for the sleep-promoting effect of melatonin is not fully understood, despite the development of melatonin agonists for the treatment of sleep disorders. It is often assumed that melatonin exerts its effects on sleep through two high-affinity, G-protein-coupled receptors, MT_1 and MT_2, though until recently, it was not known which subtype is implicated in the sleep-promoting action. IIK7, a selective MT_2 receptor agonist with approximately 90-fold higher affinity for MT_2 than MT_1, promotes sleep in rats, suggesting the effects of melatonin on sleep are mediated through the MT_2 receptor (Fisher and Sugden 2009). A more recent study further confirmed a role for the MT_2 receptor in the sleep-promoting mechanism of melatonin (Ochoa-Sanchez et al. 2011). They administered UCM765, a novel partial MT_2 receptor ligand which was effective at promoting NREM sleep in wild-type and MT_1 receptor knockout mice but not in mice lacking the MT_2 receptor. In addition, pharmacological antagonism of MT_2 receptors prevented the sleep-promoting effects of UCM765, which was shown to activate neurons expressing MT_2 receptors in the reticular thalamic nucleus (Ochoa-Sanchez et al. 2011). The analysis of the sleep in MT_1 and MT_2 deficient mice in this study revealed a complex phenotype that certainly warrants further investigation, particularly since removal of endogenous melatonin in rats has little or no effect on total sleep time or sleep/wake cycle regulation (Fisher and Sugden 2010; Mendelson and Bergmann 2001).

6 Regulation of Sleep by Social Cues

Unlike the homeostatic, circadian and photic mechanisms, the role of social cues in the regulation of sleep is more poorly understood. Due to the methods used to study sleep, animals are typically singly housed. However, a number of studies have addressed the impact of social cues on the regulation of sleep, and from this work, it appears that social interaction plays an often overlooked role in the regulation of sleep. Studies on social stimuli have been used to evaluate the effects of the quality of wakefulness on subsequent sleep (Meerlo and Turek 2001). Social conflict, where male mice were placed with an aggressive dominant male for 1 h in the middle of the light phase, produced dramatic effects on subsequent NREM sleep. EEG SWA, indicative of NREM sleep intensity, was significantly increased for 6 h and the effects on NREM sleep duration lasted for 12 h. REM sleep was suppressed during the subsequent light phase after the encounter, followed by a recovery-phase rebound. By contrast, sexual interaction, where male mice were placed with an oestrous female, produced only mild suppression of both NREM and REM sleep following the interaction. Blood sampling in this study suggested that an elevation in corticosterone may account for the temporary suppression of REM sleep (Meerlo and Turek 2001). Further studies in rats have shown that a similar social defeat model produces increases in EEG SWA, suggesting that this acute stress may increase the rate of sleep debt accumulation (Meerlo et al. 1997). To test this hypothesis, subsequent sleep deprivation was employed. Animals underwent either 1-h social defeat with 5-h sleep deprivation or 6 h sleep deprivation with no social defeat. EEG SWA was found to be higher following social defeat, illustrating that in addition to the duration of wakefulness, what is experienced during waking also modulates sleep intensity (Meerlo et al. 2001). A recent study assessed the impact of social context on sleep deprivation and EEG SWA in C57BL/6J mice (Kaushal et al. 2012). They found that that socially isolated mice exhibited a blunted homeostatic response to sleep deprivation compared to paired mice which was associated with higher anxiety levels.

Studies on environmental enrichment have suggested that rats housed in highly enriched cages exhibit longer bouts of sleep (Abou-Ismail et al. 2010), although EEG validated behaviour was not assessed in this study. An earlier study (Mirmiran et al. 1982) showed that juvenile rats raised in enriched conditions showed increased sleep time and shorter sleep latency when compared with animals housed under standard or isolated rearing conditions. Whilst limited, these studies suggest that the nature of the waking experience has a large impact on the modulation of subsequent sleep.

Earlier work by Michaud et al. (1982) found that the total amount of NREM and REM sleep was decreased when rats were placed into a novel individual cage. Other studies in mice have examined the effect of two types of environmental novelty on activity and sleep in mice. A cage change or the introduction of novel objects increased activity and NREM sleep onset latency and decreased both NREM and REM sleep time (Tang et al. 2005). The effects were relatively long-lasting with reductions in NREM sleep reported for up to 3 h after changing cages (Tang et al. 2005).

Changes in the sleeping environment can also produce significant alterations in human sleep behaviour. This is classically termed the 'first-night effect' which can be observed in individuals on the initial night of exposure to the unfamiliar surroundings of a sleep laboratory (Le Bon et al. 2001). This response to a novel environment results in an increase in arousal and vigilance characterised by an increase in NREM and REM sleep latencies together with a moderate reduction in REM sleep and a decrease in overall sleep efficiency (Shamir et al. 2000).

7 Practical Applications

The sleep/wake cycle is a complex physiological process, controlled by both circadian and homeostatic mechanisms. In addition, sleep is also regulated by the light/dark cycle, melatonin and social timing. These interactions may be summarised as a conceptual model as shown in Fig. 3, in which these internal and external mechanisms interact to modulate overt sleep behaviour. Finally, we will consider the role of sleep in two specific research areas which have broader implications for research beyond the circadian field. In addition, the reader is directed to a recent review summarising the links between clock genes and sleep and their relevance to energy metabolism, neuronal plasticity and immune function (Landgraf et al. 2012).

7.1 Sleep and Mental Health

Due to the number of brain regions and neurotransmitters involved in the regulation of sleep, it is becoming increasingly apparent that abnormal sleep is a significant comorbidity in many neuropsychiatric and neurodegenerative diseases (Wulff et al. 2009, 2010). These findings have widespread implications, not least that disturbances in mood, cognition, metabolism and social interaction may be further exacerbated by disturbances in sleep. In addition, the abnormal neurotransmitter release, stress-axis activation and medication may further destabilise the sleep/wake cycle. The complex interaction between mental health disorders and sleep is not well understood. However, it has been proposed that stabilisation of sleep in psychiatric and neurodegenerative disease may be an important means by which the devastating symptoms of these conditions may be ameliorated (Wulff et al. 2010). Clock genes have also been linked to human psychiatric disorders, and mutations have been associated with altered affective behaviour in animal models (Rosenwasser 2010). Arguably the best example of this is the *Clock*-mutant mouse, which has been proposed as a model for mania. *Clock*-mutant animals display hyperactivity, decreased sleep, lowered depression-like behaviour, lower anxiety and an increase in reward-oriented behaviour (Roybal et al. 2007). In addition,

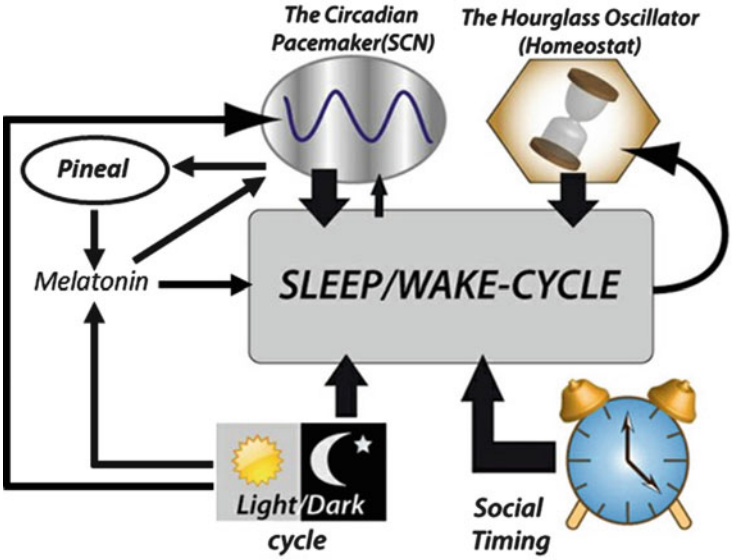

Fig. 3 Diagram illustrating the key components in the generation and maintenance of the sleep/wake cycle. Sleep is regulated by two broad mechanisms involving both the 24-h body clock (circadian system, known as process C) and a wake-dependent homeostatic build-up of sleep pressure (also called process S). The circadian pacemaker located within the suprachiasmatic nuclei (SCN) coordinates the timing of wakefulness throughout the day and sleep during the night. This 24-h rhythm interacts with the homeostatic drive for sleep, whereby the sleep pressure increases during wake and dissipates during sleep. This process has been likened to an 'hourglass oscillator'. The circadian and homeostatic drivers regulate the multiple neurotransmitter and brain systems involved in sleep and arousal. Sleep/wake behaviour in turn feeds back upon the circadian pacemaker and homeostat. These components are modulated by light which acts to entrain the circadian pacemaker to the environmental light/dark cycle, acutely suppress melatonin production from the pineal and acutely elevate or suppress levels of arousal. Finally, social activities will also modulate sleep/wake activity

disrupted circadian rhythms have recently been described in a mouse model of schizophrenia, the Bdr mutant. This mutation affects synaptosomal-associated protein (Snap)-25 exocytosis, resulting in schizophrenic endophenotypes that are modulated by prenatal factors and reversible by antipsychotic treatment (Oliver et al. 2012). These findings further suggest a mechanistic link between sleep and circadian rhythm disruption and neuropsychiatric disease (Pritchett et al. 2012). Researchers working in the mental health field should be aware that sleep disturbances are often prevalent in this patient population. Due to the common neurotransmitter systems underlying these conditions, even animal models may display disturbances in sleep and arousal which may affect the outcome of behavioural research.

7.2 Behavioural Testing

Behavioural testing in rodents is widely used in neuroscience research. One factor that is typically overlooked in many routine phenotyping assays is the state of arousal of the animals being tested. Increased arousal results in enhanced performance, up to a peak, beyond which a deterioration of performance occurs, as described by the Yerkes–Dodson Law (Yerkes and Dodson 1908). As such, animals with a low level of arousal will perform at a lower level, whereas those with a higher state of arousal will always outperform their controls. Altered states of arousal may arise from changes in homeostatic sleep drive (and previous sleep history) as well as differences in alertness due to circadian rhythm disturbances. In addition, differences in photosensitivity or responsiveness to stress (such as handling) may also give rise to altered states of arousal. As a result, behavioural testing should take into account both the sleep and circadian phenotype, including differences due to background strain (Franken et al. 1999). Furthermore, the prevailing light environment and retinal integrity as well as social and environmental modulation of arousal should all be considered (Peirson and Foster 2011). Failing to account for the state of arousal during behavioural testing can give rise to misleading results, where differences in performance are simply due to the differences in arousal state between control and experimental animals.

8 Conclusions

Whilst great advances have been made in our understanding of the circadian control of sleep via clock gene transgenics, understanding the homeostatic regulation of sleep remains an area of much ongoing research. Details of the mechanisms underlying the regulation of sleep by the light environment and social interaction also remain poorly defined. In addition to understanding the role of these various processes independently, future work will need to determine their relative contribution under natural conditions to enable us to truly understand the mechanisms influencing and giving rise to sleep and wakefulness.

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Daily Regulation of Hormone Profiles

Andries Kalsbeek and Eric Fliers

Abstract The highly coordinated output of the hypothalamic biological clock does not only govern the daily rhythm in sleep/wake (or feeding/fasting) behaviour but also has direct control over many aspects of hormone release. In fact, a significant proportion of our current understanding of the circadian clock has its roots in the study of the intimate connections between the hypothalamic clock and multiple endocrine axes. This chapter will focus on the anatomical connections used by the mammalian biological clock to enforce its endogenous rhythmicity on the rest of the body, using a number of different hormone systems as a representative example. Experimental studies have revealed a highly specialised organisation of the connections between the mammalian circadian clock neurons and neuroendocrine as well as pre-autonomic neurons in the hypothalamus. These complex connections ensure a logical coordination between behavioural, endocrine and metabolic functions that will help the organism adjust to the time of day most efficiently. For example, activation of the orexin system by the hypothalamic biological clock at the start of the active phase not only ensures that we wake up on time but also that our glucose metabolism and cardiovascular system are prepared for this increased activity. Nevertheless, it is very likely that the circadian clock present *within* the endocrine glands plays a significant role as well, for instance, by altering these glands' sensitivity to specific stimuli throughout the day. In this way the net result of the activity of the hypothalamic and peripheral clocks ensures an optimal

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endocrine adaptation of the metabolism of the organism to its time-structured environment.

Keywords Hypothalamus • Autonomic nervous system • Orexin • Glucose • Melatonin • GABA • Liver • TSH

Abbreviations

ACTH	Adrenocorticotrophic hormone
ANS	Autonomic nervous system
AVP	Arginine vasopressin
AVPV	Anteroventral periventricular nucleus
BAT	Brown adipose tissue
CLOCK	Circadian locomotor output cycles kaput
CNS	Central nervous system
CRH	Corticotrophin-releasing hormone
CSF	Cerebrospinal fluid
D2	Type 2 deiodinase
DMH	Dorsomedial nucleus of the hypothalamus
E	Oestrogen
ER	Oestrogen receptor
FFA	Free fatty acid
GABA	Gamma-aminobutyric acid
GnIH	Gonadotropin-inhibitory hormone
GnRH	Gonadotropin-releasing hormone
HPA	Hypothalamo–pituitary–adrenal
HPG	Hypothalamo–pituitary–gonadal
HPT	Hypothalamo–pituitary–thyroid
HSL	Hormone-sensitive lipase
ICU	Intensive care unit
ICV	Intracerebroventricular
IML	Intermediolateral column
L/D	Light/dark
L/L	Light/light, i.e. constant light
LH	Luteinising hormone
LM	Light microscopy
LPL	Lipoprotein lipase
MPOA	Medial preoptic area
NAMPT	Nicotinamide phosphoribosyltransferase
NPFF	Neuropeptide FF
NPY	Neuropeptide Y
OVX	Ovariectomy
PACAP	Pituitary adenylate cyclase-activating polypeptide

PBEF	Pre-B-cell colony-enhancing factor
PeN	Periventricular nucleus
pePVN	Periventricular PVN
Per	Period
PF	Perifornical area
PRV	Pseudo rabies virus
PVN	Paraventricular nucleus of the hypothalamus
Ra	Rate of appearance
RFRP	RF-amide-related peptide
RHT	Retinohypothalamic tract
SCG	Superior cervical ganglion
SCN	Suprachiasmatic nucleus
SEM	Standard error of the mean
SON	Supraoptic nucleus
subPVN	Subparaventricular PVN
T2DM	Type 2 diabetes mellitus
T3	Triiodothyronine
T4	Thyroxine
TH	Tyrosine hydroxylase
TRH	Thyrotrophin-releasing hormone
TSH	Thyroid-stimulating hormone
TTX	Tetrodotoxin
VIP	Vasoactive intestinal polypeptide
VMH	Ventromedial nucleus of the hypothalamus
VP	Vasopressin
WAT	White adipose tissue
ZT	Zeitgeber time

1 Introduction

The regular 24-h rotation of the earth has led to the evolution of autonomous circadian clocks in virtually all life forms, from prokaryotes to eukaryotes (Buhr and Takahashi 2013). In mammals, including the humans, the master endogenous clock is located in the brain. In the premodern world, the temporal cycles of feeding and fasting of our ancestors matched the patterns of wakefulness and sleep that corresponded with the daily periods of light and darkness. The circadian clock mechanism in the brain served to coordinate and anticipate our behaviour and metabolism according to this environmental periodicity induced by the earth's rotation. A proper entrainment of the endogenous clock mechanism to the outside world was ensured by a number of input signals, of which light, food intake and locomotor activity are still the most important ones.

The circadian or biological clock, located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, consists of several clusters of small and densely packed neurons in which various peptidergic transmitters are expressed (Moore 1996a). The entraining signals from light, feeding and locomotor activity are relayed to the SCN via direct projections from the retina, the hypothalamic arcuate nucleus and the raphe nucleus, respectively. The direct projection from the intergeniculate leaflet to the SCN seems to be an important secondary route for all three entraining signals. The afferent projections from these different brain structures use various neurotransmitters, including glutamate, PACAP, neuropeptide Y (NPY), neuropeptide FF (NPFF) and serotonin (Challet and Pévet 2003). The endogenous clock mechanism consists of interlocking transcriptional–translational feedback loops and contains genes necessary for oscillator maintenance ('core clock genes'), as well as specific clock-controlled output genes that impose their rhythmicity on the rest of the hypothalamus and beyond (Buhr and Takahashi 2013; Takahashi et al. 2008). A few of the peptidergic SCN transmitters, i.e. vasopressin (VP), vasoactive intestinal peptide (VIP), cardiotrophin-like cytokine and prokineticin-2, have already been identified as so-called clock-controlled genes (Hahm and Eiden 1998; Jin et al. 1999; Cheng et al. 2002; Kraves and Weitz 2006). Subsequently, the rhythmic output of this endogenous clock is conveyed to, among other things, endocrine systems. In this chapter we will show how the SCN uses its efferent projections to different combinations of intermediate, neuroendocrine and pre-autonomic neurons in the hypothalamus to translate its circadian activity into the rhythmic release of glucocorticoids, luteinising hormone (LH), melatonin, insulin, glucagon and leptin (Buijs and Kalsbeek 2001).

2 SCN Outputs

In 1972, it became clear that the SCN in the anterior hypothalamus is the seat of the central biological clock (Weaver 1998). Only a few years after this discovery, it was demonstrated that the SCN contains a prominent population of VP-containing neurons (Vandesande et al. 1974; Swaab et al. 1975). Due to its pronounced day/night rhythm in the cat cerebral spinal fluid (CSF) (Reppert et al. 1981, 1987), VP was soon identified as an output of the SCN. This important finding was followed by reports on VP-containing neurons in the SCN of a large variety of species, including man (Sofroniew and Weindl 1980; Stopa et al. 1984; Swaab et al. 1985; Cassone et al. 1988; Reuss et al. 1989; Goel et al. 1999; Smale and Boverhof 1999), as well as CSF VP rhythms in a number of species, including monkey, rat, guinea pig, goat, sheep and rabbit (Günther et al. 1984; Seckl and Lightman 1987; Stark and Daniel 1989; Forsling 1993; Robinson and Coombes 1993). Lesions of the paraventricular nucleus of the hypothalamus (PVN), hypophysectomy and pinealectomy were unable to eliminate this rhythm. The rhythms were even sustained

after complete isolation—by circular knife cuts—of the SCN in vivo. Only complete SCN lesions abolished the rhythm and in most cases reduced the amount of CSF VP to below detection level (Schwartz and Reppert 1985; Jolkonen et al. 1988). In addition, it was demonstrated that also in vitro the rhythmic release of VP from the SCN is maintained for several days (Earnest and Sladek 1986; Gillette and Reppert 1987). Additional studies showed an elevation, or poly-A tail elongation, of VP mRNA in the SCN during the light period (Uhl and Reppert 1986; Robinson et al. 1988). VP mRNA in the PVN and supraoptic nucleus (SON), on the other hand, showed no such diurnal fluctuations. Similar observations, i.e. pronounced daily fluctuations in the SCN, but not in the PVN and SON, were made for the extracellular concentrations of VP in the SCN, PVN and SON (Kalsbeek et al. 1995). The daily fluctuations of VP in the CSF are a result of the day/night rhythm in the firing rate of VP-containing SCN neurons (Buijs et al. 2006) and of the close proximity of the VP-containing SCN projections to the ventricular space, i.e. in the medial preoptic area (MPOA), the periventricular and subparaventricular nucleus (pePVN and subPVN), the dorsomedial hypothalamus (DMH) and the paraventricular nucleus of the thalamus. Since then, many SCN transmitters other than VP have come to be recognised (Morin et al. 2006), many of which also show a clear day/night rhythm in the amount of protein or mRNA expression in the nucleus itself. In the meantime, besides VP, also VIP has been demonstrated to be secreted in a circadian rhythm in vivo (Francl et al. 2010). Despite the clear demonstration with transplantation experiments that humoral factors suffice for reinstating circadian rhythms in locomotor activity and feeding and drinking behaviour (Drucker-Colin et al. 1984; Ralph et al. 1990; Silver et al. 1996), transplantation and parabiosis experiments have also unequivocally demonstrated that non-neuronal mechanisms do not suffice when it comes to reinstating circadian rhythms in all peripheral organs (Lehman et al. 1987; Meyer-Bernstein et al. 1999; Guo et al. 2005). Moreover, an elegant experiment by de la Iglesia et al. (2003) provided clear functional evidence for the necessity of point-to-point neural connections if neuroendocrine rhythms were to be sustained.

So where does the rhythmic information generated within the SCN go? Information on the distribution of SCN projections was initially obtained from neuroanatomical studies using tracing, immunocytochemistry, SCN lesions or a combination of these methods (Hoorneman and Buijs 1982; Watts and Swanson 1987; Kalsbeek et al. 1993a). All these studies showed that the outflow of SCN information was in fact surprisingly limited and pertained to the medial hypothalamus, in particular to target areas that contain mainly interneurons, such as the MPOA, DMH and the subPVN. Direct connections to neuroendocrine neurons (i.e. corticotrophin-releasing hormone (CRH)-, thyrotrophin-releasing hormone (TRH)-, tyrosine hydroxylase (TH)- and gonadotropin-releasing hormone (GnRH)-containing) in the PVN, arcuate nucleus and MPOA, and pre-autonomic neurons in the PVN were more scarce, but were reported as well (Vrang et al. 1995, 1997; Hermes et al. 1996; Teclemariam-Mesbah et al. 1997; Kalsbeek et al. 2000b; De La Iglesia et al. 1995; Van Der Beek et al. 1993, 1997). In the following

paragraphs we will explain how the SCN uses these neural connections to control peripheral rhythms in hormone release (Buijs and Kalsbeek 2001; Kalsbeek and Buijs 2002; Kalsbeek et al. 2006).

3 The Daily Cortisol/Corticosterone Rhythm

The medial parvocellular part of the PVN contains neuroendocrine neurons that synthesise CRH. Together they represent the major determinant of the set point of the neuroendocrine pathway known as the hypothalamo–pituitary–adrenal (HPA) axis (Watts 2005). In about half of the neuroendocrine CRH neurons, VP is co-expressed, with their axons projecting to the median eminence and releasing CRH and VP into the portal circulation to stimulate the adrenocorticotrophic hormone (ACTH)-producing cells in the anterior pituitary. ACTH, in its turn, controls the release of corticosterone through its stimulatory action on the adrenal cortex via the melanocortin receptor type 2. In the neuroanatomical tracing studies mentioned above, the PVN showed up as an important target area of the SCN. The close proximity of (VP-containing) SCN nerve endings near CRH-containing neurons in the PVN gave rise to the hypothesis that, via this projection, circadian information would be imprinted onto the HPA axis. In view of all the evidence in favour of an important role for VP in the output from the SCN, we began, in 1992, with microinfusions of VP and one of its antagonists. These first experiments demonstrated that VP released from SCN terminals has a strong inhibitory control over basal plasma corticosterone concentrations (Kalsbeek et al. 1992). Further studies on the relation between the circadian release of VP and the control of the daily rhythm in the activity of the hypothalamo–pituitary–adrenal (HPA) axis revealed that VP release in the rat DMH is important for ensuring low circulating levels of corticosterone during the first half of the light period (Kalsbeek et al. 1996c). In addition, the subsequent halt of VP release from these SCN terminals in the DMH is a prerequisite for the daily surge in plasma corticosterone before the onset of the main activity period of the nocturnal rat, i.e. the dark period (Kalsbeek et al. 1996b). The important role of VP in the propagation of output signals of the SCN into the PVN was nicely confirmed in a series of experiments using multielectrode recordings in hypothalamic brain slices (Tousson and Meissl 2004). These experiments showed that the circadian rhythm in spontaneous firing rate of PVN neurons was lost in slices from which the SCN had been surgically removed, but could be reinstated by either cocultures of SCN tissue or a rhythmic (12-h on, 12-h off) perfusion of VP. Moreover, simultaneous perfusion with a VP antagonist abolished PVN rhythms during coculture and rhythmic VP perfusion experiments, but not in intact slices. Together, these series of experiments clearly showed that VP is an important, but not the sole, SCN signal involved in the control of the daily rhythm in HPA-axis activity. Moreover, they formed the basis for the novel concept of SCN control over daily hormone rhythms as a push-and-pull or ying–yang mechanism, based upon alternating stimulatory and inhibitory inputs to the

appropriate target neurons. Several experiments in the years following have made a strong case for VIP as a second SCN transmitter involved in the control of the daily corticosterone rhythm (Alexander and Sander 1994; Loh et al. 2008), but its precise role is as yet unclear. Neuromedin U, too, has been proposed to be a stimulatory SCN signal (Graham et al. 2005).

In the case of the HPA axis, at first sight, the most likely target neurons appeared to be the CRH-containing neurons in the PVN. However, some evidence was inconsistent with this role for CRH neurons. First, a direct effect of VP on the CRH neuron would imply a clear daily rhythm in plasma ACTH concentrations, but this was not observed. Second, the observed inhibitory effect of VP was not in line with the usual excitatory effect of VP on its target neurons. Third, contrary to the expected abundant contacts between SCN-derived VP fibres and CRH neurons, only a limited number of such connections were found (Vrang et al. 1995; Buijs and Van Eden 2000). A detailed anatomical scheme incorporating all of the above and explaining our current view on the SCN control of the daily rhythm in HPA activity is shown in Fig. 1. The proposed intermediate role of the gamma-aminobutyric acid (GABA) ergic neurons in the subPVN and DMH in rats is supported by electrophysiological in vitro experiments using hypothalamic slices (Hermes et al. 2000). As the right-hand side image in Fig. 1 shows, the proposed important role for intermediate areas such as the subPVN and DMH also provides a good explanation for the mechanism behind the 12-h reversal of certain rhythms in nocturnal and diurnal species (for instance, that of HPA-axis activity) (Kalsbeek et al. 2008b), when the phase of SCN activity (including VP release) appears to be similar for nocturnal and diurnal species (Cuesta et al. 2009; Dardente et al. 2004).

An important spin-off of the above-mentioned VP experiments was the insight it provided into the outflow of SCN information to the autonomic nervous system (ANS) as an important mediator for the SCN control of peripheral organs and tissues. The mismatch between plasma ACTH and plasma corticosterone concentrations and responses made us realise that the ANS might be important for regulating the sensitivity of the adrenal cortex to ACTH. Transneuronal virus tracing from the adrenal did indeed reveal second-order labelling in PVN neurons and third-order labelling in SCN neurons (Buijs et al. 1999). The functional importance of this multi-synaptic neural connection between the SCN and the adrenal cortex for the daily rhythm in adrenal corticosterone release was proven later on by a series of adrenal microdialysis, adrenal denervation and adrenal transplantation studies (Jasper and Engeland 1994; Ishida et al. 2005; Oster et al. 2006). Recently, Horacio de la Iglesia and coworkers provided an additional piece of evidence for the two-stage control of the circadian corticosterone rhythm using their elegant splitting model. In hamsters, exposure to constant light (LL) conditions can induce ‘splitting’, which results in the circadian day doubling in frequency. In split animals, rest activity, body temperature and hormone secretion rhythms peak twice per day instead of once (Pittendrigh and Daan 1976; Pickard et al. 1984; Swann and Turek 1985). As expected, the unsplit hamsters showed a single peak of cortisol release concomitant with a single peak of ACTH release. Split hamsters, on the other hand, showed two peaks of plasma cortisol that

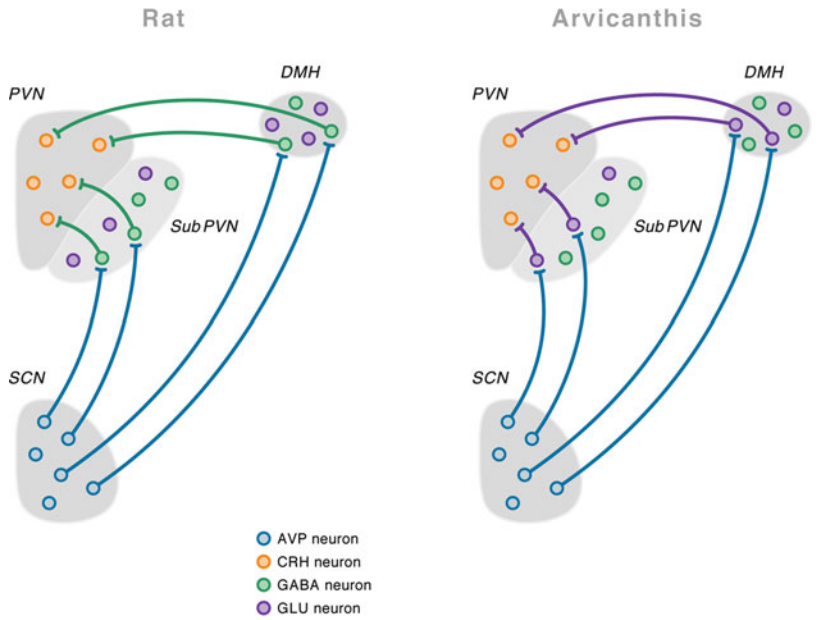

Fig. 1 Detailed anatomical scheme of demonstrated and putative connections of the suprachiasmatic nucleus (SCN) in the nocturnal rat and the diurnal *Arvicanthis ansorgei* brain to explain the opposite effects of arginine vasopressin (AVP) on the hypothalamic–pituitary–adrenal axis in these two species. AVP is released during the light period, both in the nocturnal rat and the diurnal *A. ansorgei*. In rats, AVP release during the light period will inhibit the corticotropin-releasing hormone (CRH)-containing neurons in the paraventricular nucleus of the hypothalamus (PVN) by contacting gamma-aminobutyric acid (GABA)ergic interneurons in the subPVN and dorsomedial nucleus of the hypothalamus (DMH). On the other hand, in the *A. ansorgei*, AVP release during the light period will stimulate CRH-containing neurons because it acts on the glutamatergic (GLU), instead of GABAergic, interneurons in the subPVN and DMH

were ~12 h apart but, surprisingly, did not rely on the rhythmic release of ACTH (Lilley et al. 2012). The SCN thus apparently uses a two-stage mechanism to control daily hormone rhythms: on the one hand it acts on the neuroendocrine motor neurons to influence the release of hypothalamic releasing factors, and on the other hand it also acts—through the ANS—on the target tissues to influence the sensitivity to the incoming hormonal message.

4 The Daily Melatonin Rhythm

The prime example of circadian control through the autonomic nervous system is the daily rhythm in melatonin release from the pineal gland. As early as the early 1940s, Bargman (1943) suggested that the endocrine function of the pineal gland was regulated by light, via the central nervous system. In the late 1950s, the

hormone synthesised and released by the pineal gland was identified as *N*-acetyl-5-metoxytryptamine and named melatonin by Lerner et al. (1958). The daily rhythm in pineal melatonin content, with low levels during the day and high levels during the night, was among the first hormonal rhythms to be described as a true circadian rhythm (Ralph et al. 1971; Lynch 1971). Shortly after the establishment of the SCN as the seat of the mammalian endogenous clock, a diagram was published that presented a very close approximation of the central nervous pathway controlling the circadian rhythm of pineal melatonin synthesis (Moore and Klein 1974). The pathway was unusual in the sense that it passed through both central and peripheral neural structures, i.e. contrary to the control of the daily corticosterone rhythm, which at that time only seemed to involve neuroendocrine mechanisms. The central pathway was suggested to consist of three components (1) a visual pathway transmitting information concerning environmental light intensity to the endogenous clock, (2) an output pathway of the endogenous clock transmitting its information to the spinal cord, and (3) a sympathetic pathway to the pineal gland originating from preganglionic sympathetic neurons in the intermediolateral column (IML) of the spinal cord. The early work of Kappers (1960) established the details of the peripheral sympathetic innervation of the pineal gland in the rat, whereas its functional importance was shown by Klein et al. (1971). The early works of Moore and Klein (1974) and Klein and Moore (1979) established the importance of the retinohypothalamic tract (RHT). Additional experiments involving extirpation of the superior cervical ganglion (SCG) and transection of the spinal cord established the functional importance of the sympathetic innervation (Wurtman et al. 1967; Klein et al. 1971; Bowers et al. 1984; Moore 1978; Reiter et al. 1982; Axelrod 1974; Kneisley et al. 1978). The daily rhythm in the synthesis and release of melatonin is thus ultimately controlled by the sympathetic input to the pineal gland (Moore 1996b; Drijfhout et al. 1996). A similar pathway, with potential relevance for sleep disturbances, is most likely to be present in humans (Zeitzer et al. 2000; Scheer et al. 2006). However, the details of the central pathway between the SCN and spinal cord remained enigmatic for a long time.

The first SCN lesion studies quickly proved the indispensability of the SCN for the daily rhythmicity of melatonin synthesis (Bittman et al. 1989; Moore and Klein 1974; Tessonneaud et al. 1995). Initially, it was suggested that the SCN/spinal cord pathway would involve the hypothalamic retrochiasmatic area or the lateral hypothalamus, the medial forebrain bundle and the medullary reticular formation (Klein and Moore 1979; Moore and Klein 1974). Then the first histochemical studies identified SCN projections to the PVN (Berk and Finkelstein 1981; Swanson and Cowan 1975; Stephan et al. 1981). In combination with the PVN/spinal cord projections described shortly before (Swanson and Kuypers 1980), this resulted in the identification of the PVN as an important relay for SCN output to the spinal cord (Klein et al. 1983). The key importance of the PVN as a target area for SCN information for the melatonin rhythm was corroborated by a number of subsequent neurotoxin and knife-cut studies (Bittman et al. 1989; Hastings and Herbert 1986; Lehman et al. 1984; Smale et al. 1989; Johnson et al. 1989; Pickard and Turek 1983; Badura et al. 1989; Nunez et al. 1985) and by studies involving electrical

stimulation of the PVN (Reuss et al. 1985; Olcese et al. 1987; Yanovski et al. 1987). However, it was only at the close of the twentieth century that the retrograde trans-synaptic virus tracing technique made it possible to map out the entire pathway (Larsen et al. 1998; Teclemariam-Mesbah et al. 1999). Although the viral tracing studies had helped to clearly define the total neuronal pathway, the respective roles of each of the relay stations in the control of the melatonin synthesis rhythm still remained to be determined. Besides, the different neurotransmitters used in each step of the pathway, as well as their specific daily pattern of release, remained to be brought to light.

VP became the first neurotransmitter proposed to have an inhibitory role in the control of melatonin rhythm, as it is released by the SCN with a phase which is the reverse of that of melatonin release from the pineal gland (i.e. with a peak release during the light period). Nevertheless, the first studies using VP-deficient Brattleboro rats did not reveal any difference in pineal melatonin synthesis except for the phase of the rhythm (Reuss et al. 1990; Schröder et al. 1988a). Although some studies did describe modulatory effects of the SCN transmitters VP and VIP on pineal activity, the experimental setup in these studies did not allow any definitive conclusions about the site of action of these neurotransmitters (Yuwiler 1983; Schröder et al. 1988b, 1989; Stehle et al. 1991; Reuss et al. 1990). In our own experiments, microinfusions of VP or VIP into the PVN during the initial 7 h of the dark period only produced a small stimulatory effect on plasma melatonin levels, but not the expected inhibitory effect (Kalsbeek et al. 1993b). In a later replication study, in which pineal melatonin release was used as a read-out instead of plasma melatonin, again no inhibitory effects of VP could be detected when applied at the level of the PVN (Kalsbeek et al. 2000c). On the other hand, local administration of VP in the pineal did cause a temporary increase of pineal melatonin release (Barassin et al. 2000).

In the meantime, more and more evidence pointed to the potential importance of GABA for SCN function. GABA was shown to be abundantly present in the SCN, even in projecting cells (Buijs et al. 1994; Hermes et al. 1996; Moore and Speh 1993; Okamura et al. 1989; Van Den Pol and Gorcs 1986), and we decided to test the functionality of this GABAergic output. As a first step we were able to mimic the inhibitory effect of nocturnal light exposure by administering the GABA agonist muscimol to the PVN (Kalsbeek et al. 1996a). In a follow-up study, we managed to prevent the inhibitory effect of light on nocturnal melatonin release by infusing the GABA antagonist bicuculline within the PVN, which demonstrated that light-induced inhibition of melatonin synthesis in the rat requires GABA release in the PVN (Kalsbeek et al. 1999). Next, we showed that GABA was also involved in the circadian inhibition of melatonin synthesis, independent of its role in the direct inhibitory effect of light. Indeed, blocking GABAergic transmission in the PVN during (subjective) daytime increases melatonin synthesis (Kalsbeek et al. 2000c). Based on these results and assuming an intrinsic and constant activity of the pre-autonomic PVN neurons, we proposed that the SCN controls the rhythm of melatonin synthesis by imposing an inhibitory GABAergic signal onto the PVN/pineal pathway during (subjective) daytime. However, follow-up studies conducted

in our group revealed that the melatonin rhythm obeys an even more complex power. Indeed, lesion studies comparing the effect of lesioning on melatonin synthesis of either the SCN, the PVN, or the SCG, revealed that the SCG and the PVN are just simple relay stations of SCN outputs to the pineal gland (Perreau-Lenz et al. 2003). The results of this study also proved that the rhythm of melatonin synthesis is not formed by a single circadian (daytime) inhibitory signal to the PVN but by a combination of this inhibitory signal with a stimulatory input to the PVN, also derived from the SCN. Therefore, as proposed in the foregoing for the control of corticosterone by the SCN, the SCN also seems to use multiple outputs for the control of melatonin synthesis. Looking at the mean levels of corticosterone and melatonin in SCN-lesioned animals in comparison with the peak levels in intact animals (Fig. 2), it is clear that the stimulatory part of the SCN output is even more important for the generation of the melatonin rhythm than for the generation of the corticosterone rhythm. However, the main neuronal activity of the SCN during daytime (Bos and Mirmiran 1990; Inouye and Kawamura 1979; Schwartz and Gainer 1977; Shibata et al. 1982) seems to be in clear contradiction with such a pronounced stimulatory role during the dark period. Nevertheless, it was not until 1996 that Moore noticed the apparent contradiction between the nocturnal silence of SCN neurons and the stimulation of melatonin synthesis. We tested this idea of a stimulatory role of the SCN at night in vivo (Perreau-Lenz et al. 2004), by measuring the acute effects of a temporary shutdown of the neuronal activity in the SCN on melatonin release. Nocturnal pineal melatonin release was measured by means of microdialysis before, during and after a local tetrodotoxin (TTX) application of 2 h by reverse dialysis within the SCN. This intervention resulted in an immediate diminution of melatonin secretion and an increased release of corticosterone, which shows that a generally weak neuronal activity of the SCN at night still has important physiological implications. The SCN nocturnal neuronal activity is sufficient and, more importantly, necessary to stimulate melatonin synthesis and to inhibit corticosterone at the same time. Interestingly, unlike the blockade of GABAergic transmission within the PVN (Kalsbeek et al. 2000c), TTX infusion within the SCN during daytime did not induce any increase of melatonin levels. Apparently, silencing the total neuronal activity of the SCN during daytime does not have the same effect on melatonin synthesis as does selective blocking of the SCN inhibitory transmission to the PVN. Consequently, we propose that the SCN also sustains a stimulatory output to the PVN during daytime, the final effect of which, in normal conditions, is overwhelmed by the simultaneous activity of the inhibitory GABAergic output to the PVN. Indeed, the existence of 16 % of non-rhythmic cells in the SCN (Nakamura et al. 2001) supports the idea of a tonic SCN stimulatory output originating from the same cells throughout the 24-h period. In addition, other studies showing that not all SCN neurons have the same phase of neuronal activity (Herzog et al. 1997; Nakamura et al. 2001; Saeb-Parsy and Dyball 2003; Schaap et al. 2003) suggest that the SCN could even sustain several stimulatory outputs—originating from different cell groups—at different time points.

Our ideas on the combined inhibitory and stimulatory outputs of the SCN are in agreement with studies indicating that both GABA and glutamate may be used as

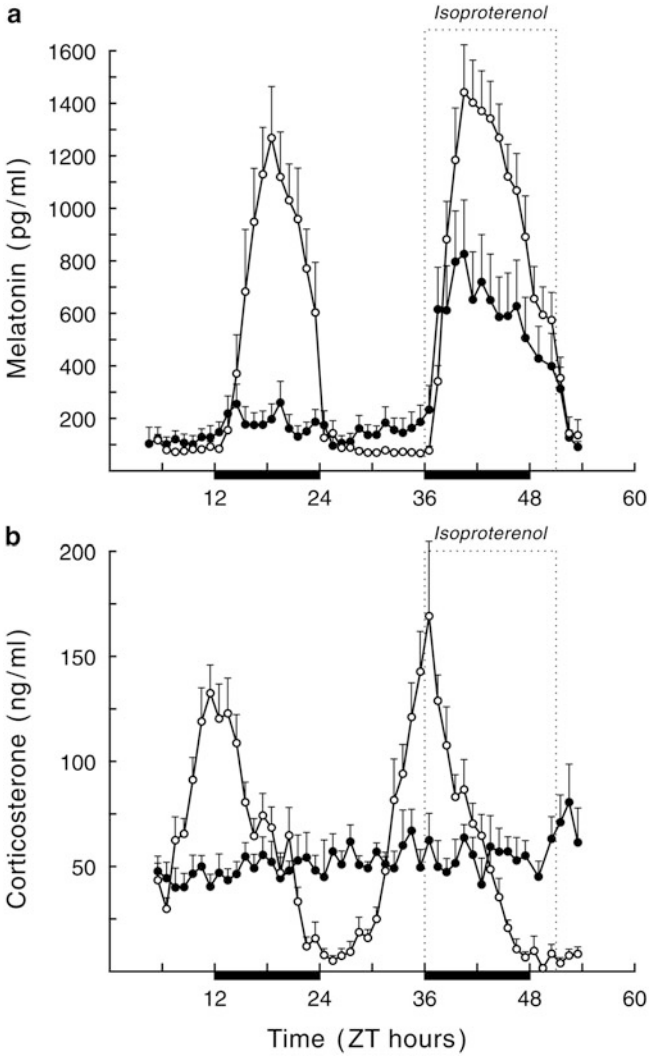

Fig. 2 Long-term secretion pattern of melatonin (**a**) and corticosterone (**b**) in intact *(open circles)* vs. SCN-lesioned (*closed circles*) rats measured by microdialysis in the pineal gland. A solution of isoproterenol was applied through the microdialysis probe in order to artificially stimulate the pineal gland and in this way check its capacity to release melatonin. The graphs represent mean data (±SEM) of eight intact and SCN-lesioned rats. (**a**) Note that the relatively low melatonin levels measured in SCN-lesioned animals increase after isoproterenol perfusion, showing (1) that the pineal is able to synthesise melatonin and (2) that the probes were correctly implanted in the gland

inhibitory and stimulatory SCN inputs, respectively, to regions of the preoptic area involved in the control of the sleep/wake rhythm (Sun et al. 2000, 2001). In addition, evidence of glutamate immunoreactivity within presynaptic boutons in the PVN (Van Den Pol 1991), as well as the demonstration of a specific glutamate

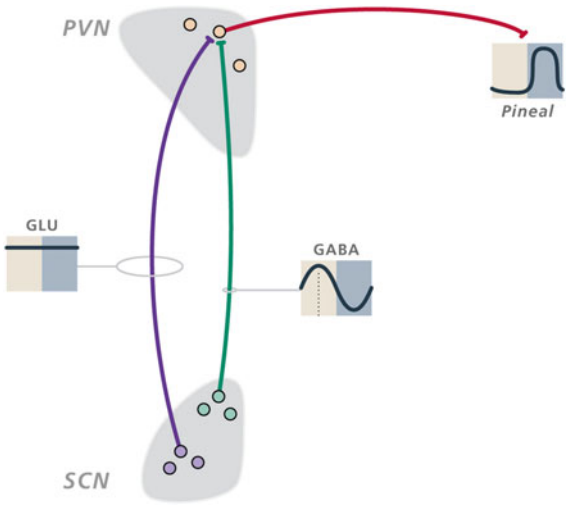

Fig. 3 Schematic presentation of the daily activity pattern of suprachiasmatic (SCN) populations of GABAergic and glutamatergic (GLU) neurons implicated in the autonomic control of the daily rhythm in pineal melatonin release. The continuous excitatory input to the sympathetic pre-autonomic neurons in the PVN from the glutamatergic SCN neurons only results in an actual activation of this neuron when the inhibitory GABAergic inhibition from the SCN is absent, i.e. the GABAergic SCN neurons function like a kind of traffic light, permitting the stimulatory input to the pre-autonomic neuron to become 'visible or noticeable' only when the GABAergic neurons permit so

release from the SCN onto (pre-autonomic) PVN neurons (Csaki et al. 2000; Cui et al. 2001; Hermes et al. 1996), shores up the idea of a glutamatergic SCN input to the PVN as well. Indeed, blocking glutamatergic transmission within the PVN at night by bilaterally infusing the *N*-methyl-D-aspartate receptor-specific glutamate antagonist, MK-801, significantly diminished melatonin levels, thus providing evidence that glutamatergic transmission within the PVN is a key player in the stimulation of melatonin at night (Perreau-Lenz et al. 2004).

In sum, the daily rhythm in plasma melatonin concentration is generated by a combination of stimulatory and inhibitory SCN outputs. The pre-autonomic PVN neurons that are in charge of the sympathetic input to the pineal gland are controlled by a combination of glutamatergic and GABAergic inputs from the SCN. The circadian and light-induced daytime activity of the GABAergic SCN projections to the PVN ensures low melatonin levels during the light period. The nocturnal arrest of the inhibitory GABAergic inputs, combined with the continuously active glutamatergic inputs, enables the pre-autonomic PVN to become active again and start a new period of melatonin synthesis and release (Fig. 3). To further define the subpopulations of SCN neurons responsible for these inhibitory and stimulatory signals, we used a combination of two different experimental paradigms, i.e. an 8-h advance of the L/D-cycle and a time-restricted feeding regime (Drijfhout et al. 1997; Kalsbeek et al. 2000a). From the results of these experiments, it became clear

that there is a small subset of *Per1*- and *Per2*-expressing neurons located in the central part of the SCN that is responsible for the nocturnal stimulation of melatonin release during the dark period (Kalsbeek et al. 2011). We propose that these neurons provide the necessary glutamatergic input to the PVN. In addition, an absence of *Per1* and *Per2* expression in the dorsal part of the SCN also seems a necessary prerequisite in order for melatonin levels to increase. We hypothesise that it is the 'activity' of these dorsal SCN neurons (and their sustained release of GABA) in the shifted animals that inhibits the pre-autonomic neurons in the PVN and prevents the reappearance of a new melatonin peak in the shifted dark period.

5 The Daily Rhythm in Luteinising Hormone Release

The SCN is important not only for the control of the daily rhythm in HPA axis and pineal gland activity but probably also for other hormonal axes, such as the hypothalamic–pituitary–gonadal (HPG) axis. Evidently, there is a clear relation between the mammalian biological clock and many aspects of reproduction: for example, the temporal organisation of pulsatile activity in the HPG axis is essential for the menstrual cycle. Lesion studies have shown that there are two brain structures that are indispensable for generating the preovulatory surge of LH: the MPOA, which contains a dense concentration of oestrogen receptors necessary for the positive oestrogen feedback, and the SCN, which provides the timing signal for the LH surge on the day of pro-oestrus. Early anatomical studies have already indicated a dense VP innervation in the MPOA, which probably derives from the SCN because it was not sensitive to gonadal hormones (Hoorneman and Buijs 1982; De Vries et al. 1984). Later studies showed that oestrogen receptor-containing neurons in the MPOA receive direct synaptic contacts from SCN fibres that probably contain VP as a neurotransmitter (De La Iglesia et al. 1995; Watson et al. 1995) and that VP receptor mRNA is expressed in MPOA neurons (Ostrowski et al. 1994; Funabashi et al. 2000a). In addition, some early studies by Södersten et al. (1983, 1985, 1986) indicated an interesting relationship between female sexual behaviour and SCN-derived VP, although at that time the effect could not be localised to a specific SCN target area. We hypothesised that the MPOA functions as an intermediate brain area for the transmission of circadian information from the SCN to the HPG axis, comparable to the intermediate function of the subPVN and DMH in the transmission of circadian information to the HPA axis. Indeed, an increase in extracellular VP levels brought about by reverse microdialysis in the MPOA of SCN-intact animals had a stimulatory effect on the LH surge, whereas it did not affect plasma corticosterone levels (Palm et al. 2001). The stimulatory effect of VP was restricted to a specific time period that coincided with the sensitive time window for a daily neuronal signal prior to the LH surge (Everett and Sawyer 1950), and also with the peak of VP secretion by SCN neurons. The important role of SCN-derived VP in the initiation of the LH surge was further emphasised by our experiments in SCN-lesioned animals. The complete absence of

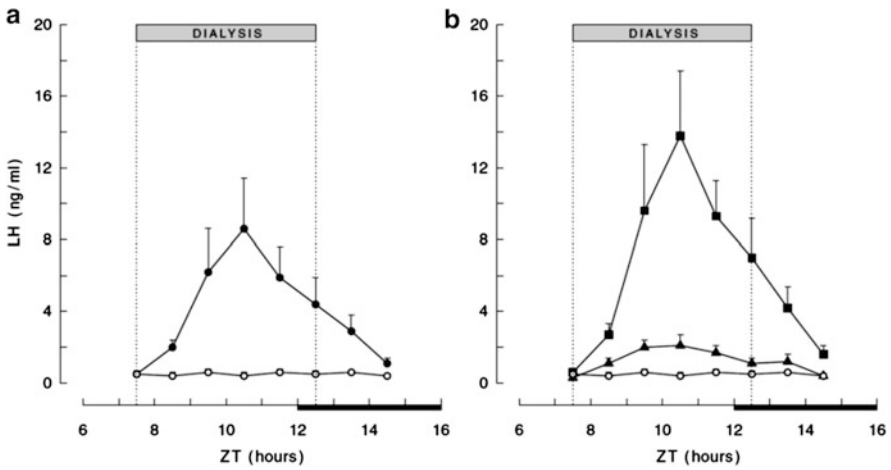

Fig. 4 Concentrations of plasma LH in SCN-lesioned, OVX + E animals. (**a**) Vasopressin-treated animals (closed symbols; $n = 9$) or vehicle-treated control animals (*open symbols*; $n = 6$), i.e. both vehicle-treated and vasopressin-treated groups consist of SCN-lesioned, OVX + E animals. In (**b**) the vasopressin-treated animals are divided in animals with a large SCN lesion (*filled triangles*; $n = 4$) and animals with a small SCN lesion (*filled squares*; $n = 5$). The smaller amount of LH released in the animals with the largest lesions probably is caused by damaged GnRH fibres travelling from the preoptic area to the median eminence, as many of these fibres travel through the perichiasmatic area bordering the SCN. Vasopressin was administered to the medial preoptic area (MPOA) via retrodialysis. The *hatched bar* represents the period of microdialysis with vasopressin; the *black bar* represents the dark period

any circadian output from the SCN induces basal, non-fluctuating LH levels, but a 2-h administration of VP in the MPOA is sufficient to reinstate a complete LH surge that is comparable to the oestrogen-induced surges in SCN-intact animals, both in shape and in amplitude (Palm et al. 1999; Fig. 4). Therefore, in our view, the high VP secretion by SCN terminals in the MPOA, occurring during the sensitive time window prior to the surge, is the circadian signal essential for the generation of an LH surge. Using completely different experimental setups, a similar conclusion was reached by Funabashi et al. (2000b) and Miller et al. (2006).

A key neuropeptide in the link between the SCN and the GnRH neurons is kisspeptin. Humans and mice lacking the kisspeptin receptor, Kiss1R (formerly known as GPR54), display hypogonadotropic hypogonadism, a condition characterised by severely impaired pubertal maturation and reproductive function due to deficient GnRH secretion (Messager 2005). Kisspeptin neurons are mostly concentrated in two discrete regions of the hypothalamus (1) rostral from the MPOA in the anteroventral periventricular nucleus (AVPV) and the rostral periventricular nucleus (PeN) and (2) arcuate nucleus in the caudal hypothalamus (Mikkelsen and Simonneaux 2009). Unlike GnRH neurons, kisspeptin-expressing neurons in the AVPV/PeN do express the alpha form of the oestrogen receptor

(ER-alpha), the subtype known to mediate the positive oestrogen feedback. Moreover, the Kiss1R is expressed in GnRH neurons (Khan and Kauffman 2011). The kisspeptin neurons thus emerge as an important link in the connection between the SCN and the LH surge. Indeed, VP-containing neurons from the SCN have been found to synapse on kisspeptin neurons. In addition, kisspeptin neurons in the AVPV/PeN express the VP receptor subtype V1a (Vida et al. 2010; Williams et al. 2011), while VIP projections to the kisspeptin neurons are scarce (Vida et al. 2010).

Another indirect connection via which the SCN could control the LH surge might involve RF-amide-related peptide (RFRP), also known as gonadotropin-inhibitory hormone (GnIH). RPRF-3-containing neurons are exclusively found in the DMH, which is one of the prime target areas of the SCN. RPRF-3 neurons express the ER-alpha and project heavily to the GnRH neurons in the MPOA. More direct evidence for a role of RFRP in the circadian regulation of the LH surge comes from 'splitting' experiments. De la Iglesia et al. (2003) had already shown that oestrogen-treated split females show an alternating activity of the left and right SCN, which went hand-in-hand with an activation of only the ipsilateral population of GnRH neurons. Later it was shown that the population of RFRP neurons in the DMH ipsilateral to the active half of the SCN shows a lower activity at the same time (Gibson et al. 2008).

Apart from this indirect control of the SCN on the LH surge via the kisspeptin neurons, direct projections from the SCN to the GnRH motor neurons, although sparse, have also been reported. Light microscopical (LM) studies, using double-labelling for SCN transmitters and GnRH, in combination with SCN lesions and tracing of SCN efferents, showed VIP-containing fibres in apposition to a substantial portion of the GnRH neurons. After lesions of the SCN, well over 50 % of the LM VIP input on GnRH neurons appeared to be derived from this nucleus (Van der Beek et al. 1993). At the ultrastructural level, too, synaptic interactions between VIP fibres and GnRH neurons were observed (Van der Beek 1996). Using immunocytochemistry for c-Fos, a marker for cell activation, a preferential activation during the initial stage of the LH surge was found of those GnRH neurons that are innervated by VIP-containing fibres (Van der Beek et al. 1994). Remarkably, these direct projections seem to be mainly VIPergic. Possibly the VIP-containing SCN projections to the GnRH neurons are involved in the transmission of the acute effects of light on the HPG axis (Van Der Beek 1996). Apposition of VP-containing fibres to GnRH neurons, although abundantly present in this area, was not observed (Van der Beek et al. 1993). The existence of a direct connection between the SCN and the GnRH system was further established by experiments using anterograde tracing and immunocytochemistry visualising GnRH at the light and electron microscopical level (Van der Beek 1996).

All in all, the circadian control of the HPG axis, using both direct and indirect connections, seems very much comparable to that summarised above for the HPA axis.

6 The Daily Rhythm in Plasma Thyroid Hormone Concentrations

Surprisingly little is known, still, about the daily rhythmicity of the hypothalamo–pituitary–thyroid (HPT) axis. Although, the daily rhythmicity of plasma thyroid-stimulating hormone (TSH) is well known in humans, neuroanatomical tracing and lesion studies in rats have shed relatively little extra light on the relationship between the central biological clock and thyroid hormone metabolism. Firstly, using immunocytochemistry SCN fibres were seen to contact TRH neurons in the PVN, a connection that may form the anatomical basis for the daily rhythms in hypothalamic TRH mRNA content (Martino et al. 1985; Collu et al. 1977; Covarrubias et al. 1988, 1994) and plasma TSH. Secondly, neuroanatomical studies using the retrograde transneuronal viral tracer PRV revealed multi-synaptic neural connections between the hypothalamic SCN and the thyroid gland via sympathetic and parasympathetic outflow. In addition, pre-autonomic neurons in the PVN, including TRH immunoreactive neurons, were labelled after injection of the PRV tracer into the thyroid gland (Kalsbeek et al. 2000b). Frequent blood sampling via permanent cannulas revealed daily rhythms of TSH and thyroid hormones with peak levels during the first half of the light period and through levels in the early dark period, which is the reverse of the human rhythm. A second peak occurred during the middle of the dark period, although this was significant only for TSH (Rookh et al. 1979; Fukuda and Greer 1975; Ottenweller and Hedge 1982; Jordan et al. 1980). A thermic ablation of the SCN completely eliminated the diurnal peak in circulating TSH and thyroid hormones, showing that the SCN drives the diurnal variation (Kalsbeek et al. 2000b). However, targeted hypothalamic infusions of SCN neurotransmitter agonists or antagonist, which had been so helpful in the above described studies on other hormonal axis, thus far have not disclosed any information on the SCN signals involved in the control of the daily HPT rhythm (unpublished data). A more recent study from our group showed a significant daily activity rhythm of the enzyme type 2 deiodinase (D2), which is the enzyme that deiodinates the prohormone thyroxine (T4) into the biologically active triiodothyronine (T3), in the pineal and pituitary gland and in the hypothalamus and neocortex. Ablation of the SCN abolished this rhythm in all brain areas studied (Kalsbeek et al. 2005). These results indicate that the bioavailability of T3 in various brain areas may show a diurnal rhythm that is driven by the SCN. However, the solid-phase liquid chromatography/tandem mass spectrometry (SPE LC-MS/MS) method—recently developed in our lab to determine thyroid hormones and their metabolites in tissue samples (Ackermans et al. 2012)—has not yet been applied to demonstrate this in brain tissue.

Using frequent blood sampling, a circadian pattern of TSH secretion was reported in humans in the early 1990s with low plasma TSH levels during the daytime, an increase in the late afternoon or early evening and a peak (the so-called nocturnal TSH surge) around the beginning of the sleep period. Plasma TSH decreases again during the later stages of sleep to reach daytime values after

morning awakening. In fact, the pronounced circadian TSH rhythm only became apparent after the inhibitory effect of sleep had been discovered (Allan and Czeisler 1994; Brabant et al. 1990). In addition to this diurnal rhythm, healthy human subjects show a clear ultradian rhythm with a pulse every 1–3 h first reported by Parker et al. (1976). Later studies using 10-min interval sampling, sensitive TSH assays and quantitative analysis confirmed that the ultradian TSH release follows a high-frequency (approx. 10 pulses per hour) and low-amplitude (0.4 mU/L) pulsatile pattern superimposed on the low-frequency and high-amplitude (1.0 mU/L) pattern of the circadian TSH rhythm. The regulatory mechanisms responsible for circadian and pulsatile TSH release in humans are incompletely understood. TSH secretion is controlled by the stimulatory action of the hypothalamic neuropeptide TRH in the PVN and the inhibitory action of central dopaminergic and somatostatinergic action, in addition to the negative hypothalamic and pituitary feedback action of the thyroid hormones T4 and T3. In spite of the clear diurnal variation in plasma TSH, circadian or sleep-related rhythms in plasma concentrations of the thyroid hormones T4 and T3 in humans are less obvious (Greenspan et al. 1986). Although this may reflect a molecular change in the TSH molecule with reduced bioactivity in the night period, altered sensitivity of the thyroid gland over the clock might be an alternative explanation as suggested by animal experimental studies (Kalsbeek et al. 2000b). On the other hand, neurologically complete cervical spinal injury did not disrupt the daily rhythmicity of TSH (or cortisol) secretion, whereas it did cause a complete loss of the plasma melatonin rhythm (Zeitzer et al. 2000).

Although there are no clear sex differences in the diurnal TSH and thyroid hormone rhythm (Roelfsema et al. 2009), contrary to what has been observed in rodents, there are several physiological (Behrends et al. 1998) and pathological conditions that alter the TSH rhythm. Bartalena et al. reported an absent nocturnal TSH surge in major depression (Bartalena et al. 1990) suggesting a role for hypothalamic TRH in the pathogenesis of HPT axis changes in depression. This was supported by the observation in a post-mortem study of markedly decreased TRH mRNA expression in the PVN of patients with major depression compared with subjects without psychiatric disease (Alkemade et al. 2003). A decreased or even absent nocturnal TSH surge was found to be present in a variety of additional nonthyroidal illnesses, occurring independently of plasma thyroid hormone concentrations or pituitary responsiveness to TRH (Romijn and Wiersinga 1990), which again may point to a hypothalamic factor. Patients with critical illness who were treated in the intensive care unit for a prolonged period of time show markedly decreased TSH pulsatility with a completely absent nocturnal TSH surge and a decreased TSH pulse amplitude (Van Den Berghe et al. 1997). The decrease in pulsatile TSH secretion was related to the low serum T3 concentration, in keeping with the concept that reduced production of thyroid hormone during prolonged critical illness may have, at least in part, a neuroendocrine origin. This was confirmed by post-mortem investigation of the PVN of patients, whose serum thyroid hormone concentrations had been assessed just before death, showing decreased TRH mRNA expression in patients with prolonged critical illness in close correlation with serum TSH (Fliers et al. 1997). Additional support for a

major role for hypothalamic TRH in the decreased TSH release during critical illness came from clinical studies in the intensive care unit (ICU) setting and showed that the continuous administration of TRH to patients with prolonged critical illness partially restored the serum concentrations of TSH as well as those of T4 and T3 (Van Den Berghe et al. 1998; Fliers et al. 2001).

In addition to critical illness, the nocturnal TSH surge is diminished in various endocrine pathologies, including hypercortisolism (Bartalena et al. 1991). A recent study in patients with primary hypothyroidism reported a persisting diurnal TSH rhythm with an earlier acrophase in most patients, while both basal and pulsatile TSH secretion rates were increased based on increased burst mass with unaltered burst frequency (Roelfsema et al. 2010). In central hypothyroidism, a lower absolute and relative nocturnal rise in TSH was observed (Adriaanse et al. 1992). Likewise, physiological conditions may affect the TSH rhythm. Clear examples are the decreased nocturnal TSH surge during fasting in association with decreased TSH pulse amplitude and unaltered TSH pulse frequency (Romijn et al. 1990) and the increased TSH surge during the first night of sleep deprivation (Goichot et al. 1998).

7 The Daily Rhythm in Plasma Glucose and Glucoregulatory Hormones

On the basis of a series of retrograde viral tracing studies from adipose tissue (both brown and white), pancreas, stomach and the heart and intestines, a similar SCN control as just discussed for the adrenal gland, pineal gland and ovaries may apply for other peripheral tissues as well (Buijs et al. 2001; Bartness et al. 2001; Scheer et al. 2001; Kreier et al. 2006), in particular for tissues involved in energy metabolism. On this basis we hypothesised that part of the action of the SCN to prepare our bodies for the alternating periods of sleep and wakefulness would be through its connections with the hypothalamic pre-autonomic neurons to control the daily setting of the sympathetic–parasympathetic balance of autonomic inputs to these peripheral organs. Indeed, in a first series of viral tracing experiments, we were able to show a clear separation of the pre-autonomic neurons that control the sympathetic and parasympathetic branch of the autonomic nervous system, up to the level of the second-order neurons in the hypothalamus (La Fleur et al. 2000; Buijs et al. 2001; Kalsbeek et al. 2004). Subsequently, we investigated whether one single group of neurons within the biological clock would be dedicated to the control of these sympathetic and parasympathetic pre-autonomic neurons, in other words, whether also within the SCN there is a clear separation of neurons controlling the sympathetic and parasympathetic branches of the autonomic nervous system. Using a combination of double viral tracing and selective organ denervation, we were able to demonstrate that the segregation of pre-sympathetic and pre-parasympathetic neurons already starts at the level of the SCN (Buijs et al. 2003). This high level of

differentiation puts the SCN in a unique position to balance the activity of both ANS branches according to the time of day. However, although these neuroanatomical data provide a nice blueprint for the possible SCN control of energy metabolism and the autonomic balance, the big question remains whether, and if so, to what extent this neuroanatomical blueprint has any functional significance.

To investigate whether the SCN control of the parasympathetic branch of the ANS is comparable to the one described above for the sympathetic branch, we then focused our attention on the daily rhythm in plasma glucose concentrations. Maintaining a constant blood glucose level is essential for normal physiology in the body, particularly for the central nervous system (CNS), as the CNS can neither synthesise nor store the glucose which is required as an energy source for the brain. The liver plays a pivotal role in maintaining optimum glucose levels by balancing glucose entry into, and removal from, the circulation. From a hypothalamic and chronobiological point of view, glucose production by the liver is especially interesting because of the clear involvement of both the sympathetic and parasympathetic input to the liver in glucose metabolism (Shimazu 1987; Nonogaki 2000; Puschel 2004) and the strong circadian control of (glucose) metabolism in the liver (Kita et al. 2002; Akhtar et al. 2002; Oishi et al. 2002). Using local intrahypothalamic administration of GABA and glutamate receptor (ant)agonists, we explored the contribution of changes in ANS activity to the daily control of plasma glucose and plasma insulin concentrations. The daily rhythm in plasma glucose concentrations turned out to be controlled according to a mechanism very much similar to the mechanism described above for the SCN control of the daily rhythm in melatonin release (Fig. 5), i.e. a combination of rhythmic GABAergic inputs and continuous glutamatergic stimulation onto liver-dedicated sympathetic preautonomic neurons in the PVN (Kalsbeek et al. 2004, 2008a). The major difference between the liver-dedicated and pineal-dedicated pre-autonomic neurons seems to be the timing of the GABAergic inputs. In the case of the pineal-dedicated preautonomic neurons, this inhibitory input is present during the major part of the light period with an acrophase around ZT6, whereas for the liver-dedicated preautonomic neurons, the acrophase of the GABAergic inhibition is somewhere around ZT2. Surprisingly, no clear evidence was found for an involvement of the parasympathetic branch of the ANS, as our previous denervation studies clearly showed the daily plasma glucose rhythm to be disrupted, also in parasympathetic liver-denervated animals (Cailotto et al. 2008).

Plasma glucose concentrations are the result of a glucose *influx* from the gut and liver and of glucose *efflux* by its uptake in brain, muscle and adipose tissue. To investigate in more detail by which glucoregulatory mechanism the just described SCN output mechanism contributes to the increased plasma glucose concentrations at awakening, we first performed a series of intravenous glucose tolerance and insulin sensitivity tests in rats. To our surprise these studies revealed that glucose tolerance and insulin sensitivity peak at the onset of the dark period (La Fleur 2003). The rise in plasma glucose concentrations at the end of the sleep period could thus not be explained by a diminished glucose uptake at this time of the L/D-cycle. These studies also indicated that glucose production should increase at the

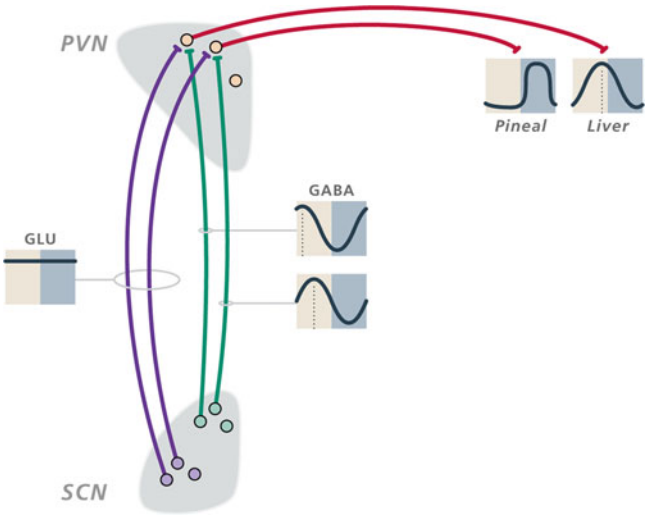

Fig. 5 Schematic presentation of the daily activity pattern of suprachiasmatic (SCN) populations of GABAergic and glutamatergic (GLU) neurons implicated in the autonomic control of the daily rhythms in pineal melatonin release and hepatic glucose production. For the control of the daily rhythms in melatonin release and glucose production, the SCN seems to rely on a uniform mechanism of continuous glutamatergic and rhythmic GABAergic inputs to the sympathetic pre-autonomic neurons. The difference in the timing of the acrophase for the melatonin and glucose production, however, indicates that separate populations of GABAergic neurons should be in contact with the pineal-dedicated and liver-dedicated pre-autonomic neurons, i.e. separate traffic lights for the pineal and the liver. In line with the idea of such a highly differentiated SCN, viral tracing studies have shown that separate neurons in the SCN are in contact with abdominal and subcutaneous adipose compartments (Kreier 2005)

end of the sleep period, to compensate for the increased glucose uptake and to explain the increased plasma glucose concentrations. We went on to combine hypothalamic infusions with systemic infusion of a stable glucose isotope. The use of the stable glucose isotope enabled us to distinguish between changes in glucose production and glucose uptake. These experiments showed that a pronounced increase in hepatic glucose production was caused by the administration of bicuculline (a GABA-A receptor antagonist) in the perifornical area lateral to the DMH and that orexin- (but not melanin-concentrating hormone (MCH)-) containing neurons in this area were strongly activated (Yi et al. 2009). Subsequent studies revealed that the hyperglycemic effect of bicuculline could be prevented by the concomitant ICV administration of an orexin antagonist and that orexin fibres impinge upon sympathetic preganglionic neurons in the IML of the spinal cord that project to the liver (Van Den Top et al. 2003; Yi et al. 2009). Earlier we had demonstrated that the hyperglycemic effect of a focal blockade of GABAergic transmission was very much dependent on the time of day (Kalsbeek et al. 2008a), indicating SCN control. Indeed, using an approach very similar to ours, Alam et al. (2005) had already demonstrated that perifornical orexin neurons are subject to an

increased endogenous GABAergic inhibition during sleep. In view of the pronounced day/night rhythm in orexin release (Zeitzer et al. 2003; Zhang et al. 2004), we hypothesised that orexin is the main connection between the biological clock and the daily rhythm in plasma glucose concentrations. To test this hypothesis, we measured the rate of glucose appearance (Ra) in ad libitum fed animals during the second half of the light period and the first hours of the dark period, i.e. during the ascending phase of the daily rhythm in plasma glucose. We combined these measurements with the ICV infusion of an orexin antagonist or vehicle. The results of this experiment pointed to an important role for the orexin system in the control by the biological clock over daily glucose homeostasis, as the ICV orexin antagonist prevented the daily dusk time increase in glucose appearance. The perifornical orexin neurons thus seem to transduce the rhythmic GABA and glutamatergic signals emanating from the SCN into a daily activation of the sympathetic input to the liver, which results in an increased hepatic glucose production at the end of the sleep period in anticipation of a new period of wakefulness (Fig. 6). Remarkably, a recent study by Shiuchi et al. (2009) demonstrated that orexin is able to stimulate glucose uptake in muscle via the ventromedial nucleus of the hypothalamus (VMH) and the sympathetic nervous system. Thus, orexin might be an important link in the SCN-controlled concomitant increase of both glucose production and glucose uptake at the onset of the activity period (La Fleur 2003). Together, these results indicate that, due to a disinhibition of the orexin system at the end of the light period, the SCN not only promotes arousal but also causes an increase of endogenous glucose production to ensure adequate concentrations of plasma glucose when the animal wakes up. Other studies made it very likely that the rhythmic activity of the orexin system is also involved in the increased activity of the cardiovascular system at awakening (Shirasaka et al. 1999; Zhang et al. 2009).

As has become evident from the daily variation in meal-induced insulin responses (Kalsbeek and Strubbe 1998), intestinal glucose uptake (Houghton et al. 2006), respiratory functioning (Bando et al. 2007) and markers of cardiac vagal activity (Burgess et al. 1997; Hilton et al. 2000; Scheer et al. 2004a), the parasympathetic branch of the autonomic nervous system too is governed by the circadian timing system. Using intra-hypothalamic infusions, we were able to show that the daily changes in the activity of the parasympathetic pre-autonomic neurons also involve a combination of GABAergic and glutamatergic inputs (Kalsbeek et al. 2008a). The inhibition of pre-autonomic neurons, both sympathetic and parasympathetic, by a daily rhythm in GABA release from SCN efferents to the PVN turned out to be a general principle. However, a major difference between the circadian control of parasympathetic and sympathetic pre-autonomic neurons appears to be the origin of the excitatory glutamatergic inputs. SCN-lesion studies proved that the excitatory input to the sympathetic pineal-dedicated pre-autonomic neurons was derived from the SCN neurons (Perreau-Lenz et al. 2003), but also that the glutamatergic inputs to the parasympathetic pancreas-dedicated pre-autonomic

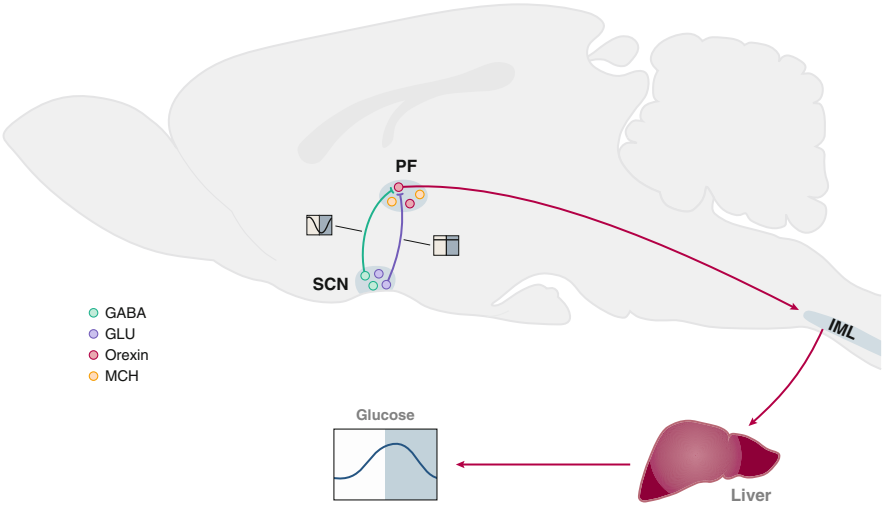

Fig. 6 Midsagittal view of the rat brain with a hypothesised presentation of the involvement of orexin neurons in the autonomic control of the daily rhythm of hepatic glucose production. (1) The orexin-containing neurons in the perifornical area (PF) are innervated by both glutamatergic and GABAergic projections from the biological clock (SCN). During the main part of the light period, activation of the orexin neurons by the excitatory glutamatergic inputs is prevented by releasing the inhibitory neurotransmitter GABA (the daily activity pattern of these inputs is indicated by the *lines* in the *yellow/blue boxes* aside the projections). The circadian withdrawal of the GABAergic input allows the orexin neurons to become active at the onset of darkness. (2) Subsequently, the excitatory effect of orexin on the preganglionic neurons in the IML of the spinal cord will (3) activate the sympathetic input to the liver and result in increased hepatic glucose production. Orexin also stimulates glucose uptake in skeletal muscle via action in the VMH and mediated through the sympathetic nervous system (Shiuchi et al. 2009); but, as it is not clear yet how the message is propagated from the VMH to the autonomic nervous system, this action has not been incorporated in this scheme

neurons cannot be derived from SCN neurons (Strubbe et al. 1987). At present, it is not yet clear from which extra-SCN source the glutamatergic inputs to the parasympathetic pancreas-dedicated pre-autonomic neurons originate, but likely candidates are the VMH and arcuate nucleus (Fig. 7).

8 Daily Rhythms in Plasma Adipokines

Adipose tissue composes one of the largest organs in the body. It can make up from 5 % of body weight in lean men to over 50 % in the morbidly obese. In mammals, two major, functionally different, types of adipose tissue have been described: brown adipose tissue (BAT) and white adipose tissue (WAT). BAT and WAT share the ability to store lipids as triglycerides, but use them for different purposes. BAT produces heat and plays an important role in non-shivering thermogenesis.

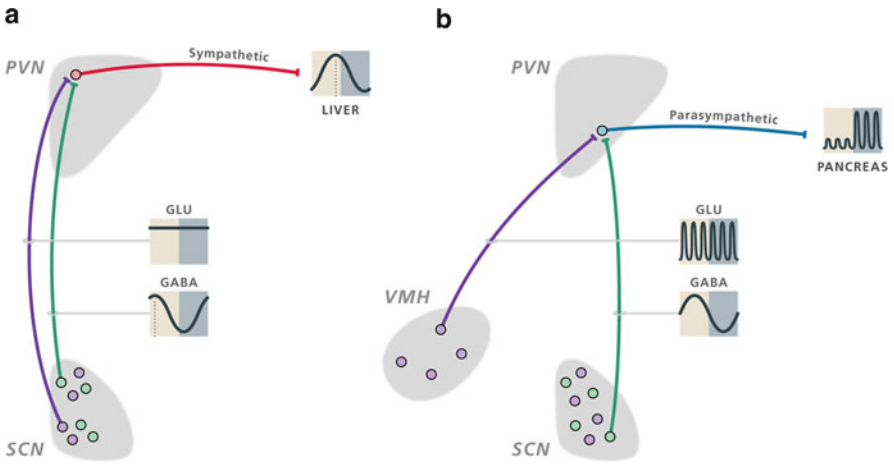

Fig. 7 Schematic presentation of the daily activity pattern of hypothalamic populations of GABAergic and glutamatergic (GLU) neurons implicated in the autonomic control of the daily rhythms in hepatic glucose production (*left-hand side*) and feeding-induced insulin release (*right-hand side*). Similar to the previously proposed circadian control of the *sympathetic* pre-autonomic neurons (*left-hand side*), also the circadian control of the *parasympathetic* pre-autonomic neurons seems to rely on a combination of glutamatergic and GABAergic inputs (*right-hand side*). However, whereas for both types of neurons the rhythmic GABAergic input is derived from the SCN, the sources of glutamatergic input seem to be different, i.e. SCN for the *sympathetic* pre-autonomic neurons and extra-SCN for the *parasympathetic* ones. In the figure the VMH is indicated as the most likely origin of the glutamatergic input, but at present experimental evidence is lacking for this proposition. Moreover, whereas the glutamatergic input from the SCN to the *sympathetic* pre-autonomic neurons is proposed to be continuous, the glutamatergic input from the VMH to the *parasympathetic* pre-autonomic neurons is proposed to be dependent on feeding activity

WAT, besides functioning as mechanical and thermal protection of vital organs and as an important long-term energy store, secretes several proteins that influence processes as diverse as haemostasis, blood pressure, immune function, angiogenesis and energy balance (Christodoulides et al. 2009).

Obesity is characterised by excessive accumulation of triglycerides in adipose tissue, determined by a net balance of fatty acid uptake and release in favour of fat storage over fat mobilisation. The rich innervation of adipose tissue by sympathetic fibres is well known, and activation of these fibres is associated with enhanced lipolysis (Weiss and Maickel 1968). Until a few years ago, it was thought that parasympathetic innervation of adipose tissue did not occur and that lipogenesis was merely controlled by hormones, the mass action of free fatty acids and sympathetic withdrawal. In view of the importance of this balance between lipogenesis and lipolysis, and the capacity of the SCN to control the sympathetic/parasympathetic balance in other organs, we reinvestigated the existence of parasympathetic input to adipose tissue. This would allow us to test the possibility of SCN control of this lipogenesis/lipolysis balance through the autonomic nervous system. Indeed, as previously reported by others (Bamshad et al. 1998), at first we

found only very sparse parasympathetic input to white adipose tissue. However, combining the viral tracing technique with a prior selective sympathetic denervation of the targeted fat pad resulted in pronounced labelling of the parasympathetic motor neurons in the brainstem (Kreier et al. 2002). It is not clear what causes this increased visibility of the parasympathetic input, but one possibility is that the parasympathetic fibres are only exposed to the virus when the more active sympathetic fibres have been removed, as previous studies have shown that viral tracing can be modulated by neuronal activity (Lee and Erskine 2000). Although parasympathetic innervations of WAT had not been replicated by others at this stage, these observations provided the neuroanatomical substrate for earlier pharmacological observations in human microdialysis studies that showed cholinergic effects on lipolysis (Andersson and Arner 1995) and the more recent identification of functional acetylcholine receptors in rat adipocytes (Liu et al. 2004; Yang et al. 2009). In addition, our own functional studies provided clear evidence for an anabolic function of this parasympathetic innervation of adipose tissue. Euglycemic hyperinsulinemic clamp studies revealed a >30 % reduction in the insulin-mediated uptake of glucose and free fatty acids (FFAs) in adipose tissue as a result of selective removal of its parasympathetic input. Moreover, without parasympathetic input, the activity of the catabolic enzyme hormone-sensitive lipase (HSL) increased by 51 % in the denervated adipose tissue (Kreier et al. 2002). Follow-up studies using two different PRV tracers and selective denervation of the adipose tissue showed the presence of both 'sympathetic' and 'parasympathetic' adipose neurons in the hypothalamus, including the SCN (Kreier 2005). These results thus provide clear evidence that the SCN may use the ANS also to enforce its day/night rhythms upon the endocrine and metabolic functioning of adipose tissue.

As indicated above, white adipose tissue also plays a central role in the regulation of energy metabolism, mainly via the secretion of factors (adipokines) that regulate appetite, food intake, glucose disposal and energy expenditure (Wang et al. 2008). Adipokines are secreted by adipocytes and/or the stromavascular fraction of WAT. Originally the term adipokine was proposed to describe cytokines secreted from adipocytes specifically. However, as many cell types in adipose tissue have been found to secrete proteins and other proteins besides cytokines are being produced, the term adipokine is now widely used to describe proteins secreted from adipose tissue (Stryjecki and Mutch 2011; Wang et al. 2008). Extensive reviews on the metabolic functions of adipokines have been published in recent years (Halberg et al. 2008; Trujillo and Scherer 2006; Poulos et al. 2010; Maury and Brichard 2010). Like most other tissues, WAT gene expression shows circadian rhythmicity (Ando et al. 2005; Ptitsyn et al. 2006). Indeed, both fat deposition by the key enzyme lipoprotein lipase (LPL) and fat mobilisation by HSL show a clear daily rhythm in the white adipose tissue of humans and laboratory animals (Hems et al. 1975; Cornish and Cawthorne 1978; Bergö et al. 1996; Hagström-Toft et al. 1997; Benavides et al. 1998). Moreover, also the circulating plasma levels of a number of adipokines, including leptin, as well as their adipose mRNA levels show clear day/night rhythms.

Leptin is a hormone secreted by adipose tissue in proportion to body fat amount and relays fat storage information to the brain. High levels of leptin signal satiety and reduce food intake, whereas low levels of leptin stimulate food intake (Schwartz et al. 2000). The discovery that plasma leptin helps to regulate body weight through its hypothalamic effects on food intake and energy expenditure represented a major breakthrough in our understanding of the neuroanatomical and molecular components of the systems involved in energy homeostasis (Farooqi 2011). For the discovery of this previously unknown endocrine system, Coleman and Friedman received the Albert Lasker Award for Basic Medical Research in 2010 (Flier and Maratos-Flier 2010).

Many studies by now have shown that plasma levels of leptin fluctuate over the day and night (Simon et al. 1998; Kalsbeek et al. 2001; Gavrila et al. 2003; Shea et al. 2005). It has been shown that plasma leptin levels are regulated not only by fat mass and the biological clock but also by feeding and that long periods of fasting eliminate the leptin rhythm (Elimam and Marcus 2002). However, under constant and continuous feeding conditions, a circadian rhythm in leptin persists, indicating a role for the circadian clock in regulating leptin levels during fed conditions (Simon et al. 1998; Kalsbeek et al. 2001). In healthy volunteers, misalignment between behaviour and endogenous circadian timing leads to lower overall leptin levels (Scheer et al. 2009), suggesting that leptin responds to the endogenous circadian clock independent of behavioural factors such as feeding. Although SCN lesions eliminate leptin circadian rhythmicity (Kalsbeek et al. 2001), cultured adipocytes still show rhythmic leptin mRNA expression, implying regulation by an endogenous clock within the adipocytes (Brown and Azzi 2013; Bass 2013; Otway et al. 2009). In addition to being regulated by the clock, leptin may also serve as an input factor for the biological clock. The leptin receptor is expressed in SCN cells, and in vitro leptin can phase-advance the SCN (Prosser and Bergeron 2003). In sum, leptin is a pivotal factor in the interplay between feeding cues, metabolic state and circadian timing.

Besides leptin also several other adipokines show a significant day/night rhythm. Adiponectin is an adipokine that is involved in glucose and lipid metabolism by increasing fatty acid oxidation and potentiating insulin-mediated inhibition of hepatic gluconeogenesis, thus promoting insulin sensitivity (Barnea et al. 2010). Interestingly, although adiponectin is produced by adipose tissue, its serum levels and WAT gene expression decrease in obesity and in animals fed a high-fat diet (Barnea et al. 2010; Boucher et al. 2005; Turer et al. 2011). Both in vitro and in vivo, adiponectin has a significant day/night rhythm (Scheer et al. 2010; Barnea et al. 2010; Otway et al. 2009; Gavrila et al. 2003; Garaulet et al. 2011), with a trough at night for humans, and during the day for rats (Scheer et al. 2010; Oliver et al. 2006). This rhythm is not driven by feeding/fasting cycle in lean men (Scheer et al. 2010). $Clock^{\Delta 19}$ mutant mice that retain melatonin rhythmicity ($Clock^{\Delta 19}$ + MEL) show increased eWAT adiponectin gene expression, which may contribute to the improved insulin resistance that is found in $Clock^{\Delta 19}$ + MEL mice compared to $Clock^{\Delta 19}$ mice (Kennaway et al. 2011).

Resistin is a cytokine that is produced in WAT (adipocytes in rodents, macrophages in human) and is a potential mediator of type 2 diabetes and cardiovascular disease (Ando et al. 2005; Oliver et al. 2006; Rajala et al. 2004; Schwartz and Lazar 2011) with higher expression rates in omental versus subcutaneous WAT of obese female subjects (Fain et al. 2003). Resistin mRNA expression is rhythmic in several WAT compartments in rats, with a peak in the late dark/early light phase (Oliver et al. 2006). Resistin is downregulated by fasting and upregulated by (re-) feeding (Oliver et al. 2006). However, WAT gene expression levels of resistin are decreased in obese and high-fat-diet-fed mice (Boucher et al. 2005). Rotating shift workers have elevated plasma levels of resistin compared to day work controls (Burgueño et al. 2010).

Visfatin is a multifunctional protein produced by adipose tissue, but also by skeletal muscle, liver, and immune cells, and is also known as nicotinamide phosphoribosyltransferase (Nampt) or pre-B-cell colony-enhancing factor (PBEF). Circulating visfatin levels have been reported to be elevated in type 2 diabetes and obesity (Fukuhara et al. 2005; Chen et al. 2006; Hallschmid et al. 2009; Berndt et al. 2005). In rodents the expression of visfatin shows a circadian rhythm in WAT, as well as in adipocytes and hepatocytes (Ando et al. 2005; Ramsey et al. 2009), but also circulating levels in human plasma show a clear daily rhythm (Benedict et al. 2012). Since plasma visfatin levels also seem to be affected by sleep duration (Hayes et al. 2011; Benedict et al. 2012), visfatin has been proposed to have a regulatory role in the deleterious metabolic effects of sleep deprivation.

9 Future Perspective

It is now generally accepted that the SCN is the principal neural structure that mediates circadian rhythms in mammals, including man. A key question at this stage is whether a strengthening of the SCN signal could alleviate pathologies such as insulin resistance, obesity and hypertension. An interesting example of the possible utility of this approach was provided by an experiment investigating this in terms of alleviating the increased blood pressure in hypertensive patients. A randomised, double-blind, placebo-controlled crossover study was conducted in which 16 men with untreated essential hypertension were treated with oral melatonin (2.5 mg daily; 1 h before sleep) for 3 weeks. Repeated melatonin administration reduced ambulatory systolic and diastolic blood pressure by 6 and 4 mmHg, respectively (Scheer et al. 2004b).

A second example is provided by another ‘experiment of nature’, i.e. ageing: a marked decrease in the number of VP-containing neurons was observed in subjects between 80 and 100 years of age (Swaab et al. 1985; Harper et al. 2008). Moreover, a flattening of the daily rhythm in SCN VP abundance was already observed in subjects >50 years of age (Hofman and Swaab 1994). In addition, an age-related increase in abdominal obesity and type 2 diabetes is well known. As with the daily

melatonin treatment in hypertensive patients, long-term treatment of elderly with whole-day bright light during the day period improved cognitive and noncognitive symptoms of dementia (Riemersma-Van Der Lek et al. 2008), although at this stage no metabolic parameters were investigated.

Interestingly, the loss of immunoreactivity for SCN neurotransmitters during ageing and hypertension is probably not due to a loss of neurons, but to a decreased activity of these neurons. Therefore, an important way to revitalise a flattened and disorganised SCN output might be to enhance the rhythmic input signals to the SCN. Thus, daily melatonin treatment and daily light treatment may help to improve circadian rhythms in behaviour by an enhancement of biological clock functioning. A third treatment strategy might be daily exercise, which is indeed a very effective way to improve glucose tolerance. Although the experiments described above may yield therapeutic strategies to counteract the negative health effects of a chronically desynchronised SCN output, they may not apply for shift workers, as here the circadian misalignment is in constant flux. During working days shift workers are compelled to shift their sleep/wake rhythm to meet the needs of work hours, but during days off they revert to a normal daytime activity schedule to meet the needs of social life. Therefore, future studies identifying the exact mechanisms of internal desynchronisation are justified if we are to propose behavioural strategies capable of minimising the adverse effects of circadian misalignment (Roenneberg et al. 2013).

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Circadian Clocks and Mood-Related Behaviors

Urs Albrecht

Abstract Circadian clocks are present in nearly all tissues of an organism, including the brain. The brain is not only the site of the master coordinator of circadian rhythms located in the suprachiasmatic nuclei (SCN) but also contains SCN-independent oscillators that regulate various functions such as feeding and mood-related behavior. Understanding how clocks receive and integrate environmental information and in turn control physiology under normal conditions is of importance because chronic disturbance of circadian rhythmicity can lead to serious health problems. Genetic modifications leading to disruption of normal circadian gene functions have been linked to a variety of psychiatric conditions including depression, seasonal affective disorder, eating disorders, alcohol dependence, and addiction. It appears that clock genes play an important role in limbic regions of the brain and influence the development of drug addiction. Furthermore, analyses of clock gene polymorphisms in diseases of the central nervous system (CNS) suggest a direct or indirect influence of circadian clock genes on brain function. In this chapter, I will present evidence for a circadian basis of mood disorders and then discuss the involvement of clock genes in such disorders. The relationship between metabolism and mood disorders is highlighted followed by a discussion of how mood disorders may be treated by changing the circadian cycle.

Keywords Depression • Obesity • Light • Drugs

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1 Evidence for a Circadian Basis of Mood Disorders

Patients with depressive disorders appear to display abnormal circadian rhythmicity in a variety of body functions such as body temperature, plasma cortisol, noradrenaline, thyroid-stimulating hormone, blood pressure, and melatonin rhythms (Atkinson et al. 1975; Kripke et al. 1978; Souetre et al. 1989). Interestingly, treatment of patients with antidepressants or mood stabilizers normalizes these hampered rhythms. Furthermore, genetic alterations in casein kinases (Shirayama et al. 2003; Xu et al. 2005) modulating the circadian clock mechanism as well as polymorphisms found in clock genes have been found to associate with sleep disorders and depressive behavior [for a comprehensive list, see Kennaway (2010)]. However, most of these polymorphisms were not located in the coding region of clock genes.

Interestingly, nearly all individuals that suffer from mood disorders benefit from strict daily routines including strictly followed bedtime and rise in the morning (Frank et al. 2000). These routines probably help to maintain the circadian integrity of the body (Hlastala and Frank 2006). The effect of having a clock that is out of sync with the environment is evident to anyone who has experienced jet lag after traveling (Herxheimer 2005). Such changes in timing can cause in some individuals depressive or manic episodes. This has also been observed in shift workers where some individuals will develop mood disorders over time (Scott 2000). Recent work shows that a relationship between severity of bipolar depression and circadian misalignment is likely to exist (Emens et al. 2009; Hasler et al. 2010). Hence, the inability to properly adapt to environmental change appears to contribute to the development of mood disorders such as depression.

One of the most common disorders due to improper adaptation to changes in the environment is seasonal affective disorder (SAD). It is characterized by depressive symptoms that occur only during the winter months (Magnusson and Boivin 2003). It is hypothesized that melatonin, a circadian hormone secreted by the pineal gland, is involved in the development of SAD (Pandi-Perumal et al. 2006). Although it is clear that melatonin participates in the regulation of sleep and can be suppressed by light, it is still controversial whether a link between melatonin rhythms and SAD exists. Another equally controversial hypothesis to explain SAD is the circadian phase shift hypothesis, which is based on the observation that application of early morning bright light is effective in treating SAD (Lewy et al. 1998; Terman and Terman 2005) probably due to phase advancing the circadian system putting it back in sync with the sleep/wake cycle.

The mechanism underlying the association between circadian rhythms and mood disorders is unknown. It is conceivable, however, that molecular clock components may affect the expression of neurotransmitters and their receptors. It is of note that some of the major neurotransmitters, such as serotonin, noradrenaline, and dopamine, display a circadian rhythm in their levels (Weiner et al. 1992; Castaneda et al. 2004; Weber et al. 2004; Hampp et al. 2008). Also circadian rhythms in the expression and activity of several of the receptors for neurotransmitters have been

observed, suggesting that the entire circuits may be under circadian clock control (Kafka et al. 1983; Coon et al. 1997; Akhisaroglu et al. 2005). Therefore, it seems likely that disruption of the normal rhythms in neurotransmitter circuits may affect mood and mood-related behavior. How the clock modulates these circuits is still uncertain but emerging (Hampp et al. 2008).

2 Circadian Clock Genes and Mood Disorders

Studies in humans have begun to identify polymorphisms in certain circadian clock genes that associate with mood disorders. The T3111C SNP of the *CLOCK* gene associates with a higher recurrence rate of bipolar depression (Benedetti et al. 2003), and it associates with greater insomnia and decreased need for sleep in bipolar patients (Serretti et al. 2003). Two other members of the molecular clock, *BMAL1* and *PER3*, have been implicated in bipolar depression (Nievergelt et al. 2006; Benedetti et al. 2008). Recent studies suggest that SNPs of *PER2*, *NPAS2*, and *BMAL1* are associated with an increased risk for SAD (Partonen et al. 2007) and *Cry2* may be associated with depression (Lavebratt et al. 2010). All these clock genes appear to be associated with bipolar disorders (BD) and lithium response (McCarthy et al. 2012). Interestingly, associations of clock gene polymorphisms have been made with other psychiatric disorders such as schizophrenia and alcoholism, suggesting that clock genes are important in a range of psychiatric conditions (Spanagel et al. 2005; Mansour et al. 2006).

Animal studies support the role of circadian clock genes in mood regulation. Clock genes are expressed in many brain areas of the rewards system, which contributes to mood regulation. These areas include the ventral tegmental area (VTA), prefrontal cortex (PFC), amygdala (AMY), and the nucleus accumbens (NAc) (Fig. 1).

In these brain structures, 24-h oscillations of clock gene expression are not necessarily in the same phase but retain a specific phase relationship to one another [reviewed in Guilding and Piggins (2007)]. Mice carrying a mutation in the *Clock* gene [*Clock*Δ19 (Vitaterna et al. 1994; King et al. 1997)] display a behavior similar to human mania, and when treated with lithium, the majority of their behavioral responses are normalized toward those of wild-type mice (Roybal et al. 2007). Interestingly, transgenic mice overexpressing GSK3β show similarities to the phenotype of *Clock* mutant mice; they are hyperactive and have reduced immobility in the forced swim test (Prickaerts et al. 2006). This indicates that lithium, which inhibits GSK3β activity, acts at least partially via this kinase in *Clock* mutant mice normalizing their behavior. Reduced mobility in the forced swim test has also been observed in *Per2* mutant mice [$Per2^{Brdm1}$ (Zheng et al. 1999)], which is accompanied by elevated dopamine levels in the NAc (Hampp et al. 2008). Taken together, these findings may suggest that various mutations in circadian clock genes result in a similar manic phenotype. However, $Per1^{Brdm1}$ and $Per2^{Brdm1}$ mutant mice are not hyperactive like *Clock*Δ19 mice. $Per1^{Brdm1}$ mutant mice show

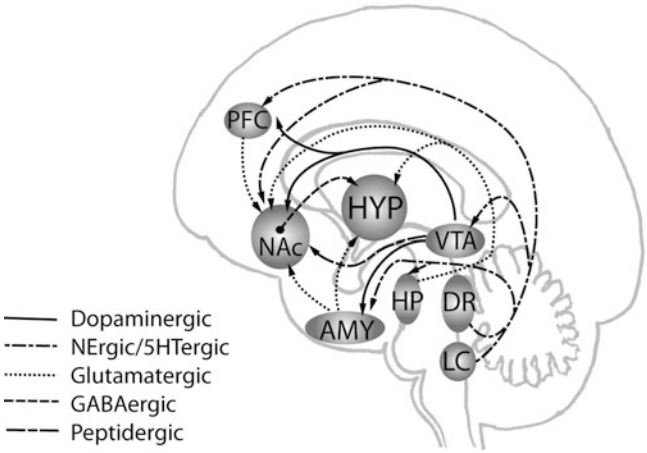

Fig. 1 Brain regions involved in mood regulation. Besides the hippocampus (HP) and the prefrontal cortex (PFC), several subcortical structures are involved in reward, fear, and motivation. These include the nucleus accumbens (NAc), amygdala (AMY), and hypothalamus (HYP). The figure shows only a subset of the many known interconnections between these various brain regions. The ventral tegmental area (VTA) provides dopaminergic input to the NAc, AMY, and PFC. *DR* dorsal raphe nuclei, *GABA* gamma-aminobutyric acid, *LC* locus coeruleus, *NE* norepinephrine, *5HT* serotonin

opposite responses to conditioned cocaine preference compared to *Clock*Δ19 and *Per2*^{Brdm1} mutant mice (Hampp et al. 2008; Abarca et al. 2002), and they show no elevated alcohol preference compared to *Per2*^{Brdm1} mutants (Spanagel et al. 2005; Zghoul et al. 2007). However, in response to social defeat, *Per1*^{Brdm1} mutants increase alcohol consumption (Dong et al. 2011) indicating that the *Per1* gene is a nodal point in gene x environment interactions. A recent study also indicates that a *Per3* promoter polymorphism is associated with alcohol and stress response (Wang et al. 2012). Overall it seems that individual members of the circadian clock mechanism may have separate functions in regulating mood- and reward-related behaviors. These functions may be residing outside the central SCN pacemaker in specific brain structures (e.g., VTA, AMY, or NAc) or in peripheral clocks (e.g., liver, gut).

In this context, it is of interest to note that *Clock* is expressed in peripheral tissues (although low expression is observed in certain brain areas) in contrast to *Npas2*, a *Clock* homologue, which is strongly expressed in the brain (see Allen Brain Atlas, http://www.brain-map.org/). Accordingly, only peripheral circadian clocks require *Clock* (DeBruyne et al. 2007a), whereas in the SCN, *Npas2* can replace *Clock* function (DeBruyne et al. 2007b). Therefore, phenotypes observed in *Clock* mutant mice may also include effects derived from lack of this gene in peripheral tissues (see below section on Metabolism).

Dopamine, an important neurotransmitter in the reward system, displays daily rhythms in its levels in the NAc (Hampp et al. 2008; Hood et al. 2010) suggesting

that the entire reward circuit may be under circadian clock influence. Consistent with this view are the observations that proteins involved in dopamine metabolism and transmission display diurnal rhythms in their expression, including tyrosine hydroxylase (TH) (McClung et al. 2005), a rate-limiting enzyme in dopamine synthesis; monoamine oxidase A (MAOA) (Hampp et al. 2008), a rate-limiting enzyme in dopamine degradation; and dopamine receptors (Hampp et al. 2008; McClung et al. 2005). When *Clock* gene expression is knocked down in the VTA, which projects to the NAc via dopaminergic neurons, an increase in dopaminergic activity is observed (Mukherjee et al. 2010). This increased dopaminergic tone results in changes in dopamine receptor (DR) levels with both D1 and D2 type of DRs augmented (Spencer et al. 2012). Interestingly, a shift of the ratio of D1:D2 receptors in favor of D2 receptor signaling was observed leading to alterations in locomotor responses to D1- and D2-specific agonists (Spencer et al. 2012). In $Per2^{Brdm1}$ mutant mice, the dopamine levels in the NAc are elevated as evidenced by microdialysis (Hampp et al. 2008). This is associated with a decrease in MAOA activity in the VTA and NAc. Interestingly, the *Maoa* gene is directly regulated by BMAL1, NPAS2, and PER2, and hence, *Maoa* is a clock-controlled gene (CCG, Fig. 2). This directly links the clock with dopamine metabolism (Hampp et al. 2008). Of note is that SNPs for *BMAL1*, *NPAS2*, and *PER2* are associated with an increased risk for SAD in humans (Partonen et al. 2007) establishing a parallel between the findings in mouse and humans.

The behavioral phenotypes observed in $Per2^{Brdm1}$ mutant mice are probably only partially due to elevated dopamine levels, because these animals also show abnormally high glutamate levels in the striatum (Spanagel et al. 2005). Therefore the balance between dopaminergic and glutamatergic signaling in the striatum of these mice appears to be deregulated. This may lead to abnormal neural phase signaling, which is a putative coding mechanism through which the brain ties the activity of neurons across distributed brain areas to generate thoughts, percepts, and behaviors (Lisman and Buzsaki 2008). In *Clock*Δ19 mutant mice, this phase signaling seems to be disturbed and is accompanied by abnormal dendritic morphology and a reduction in the levels of glutamate receptor subunit GluR1 (Dzirasa et al. 2010). Mice lacking GluR1 show behaviors related to mood disorders and respond positively to lithium (Fitzgerald et al. 2010). These observations support the notion that alterations in the balance between dopaminergic and glutamatergic signaling are probably important in the regulation of mood state and that this may involve circadian clock components. However, research linking clock genes and mood disorders is still in the early stages, and more investigations are needed to understand how the circadian clock mechanism impinges on mood regulation and thus affects depression including major depression, bipolar disorder, and seasonal affective disorder.

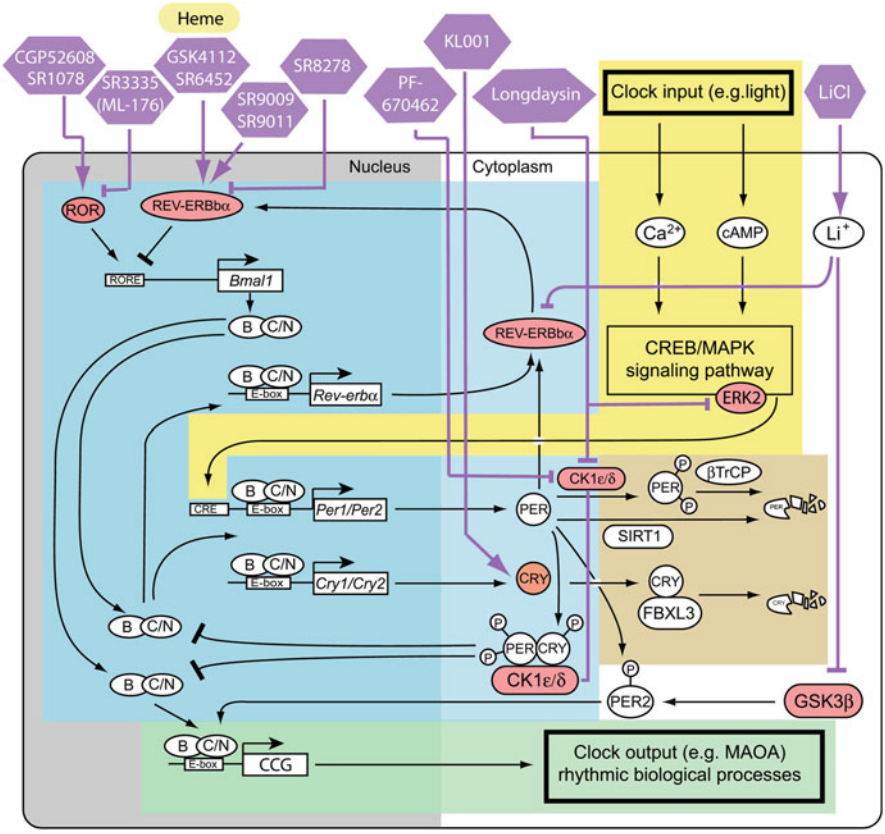

Fig. 2 Schematic representation of the mammalian circadian clock mechanism in a cell. The *blue* area depicts the autoregulatory transcriptional translational feedback loop. The transcription factors BMAL1 (B) and CLOCK (C) or NPAS2 (N) form a heterodimer which binds to E-box elements in the promoters of *Per1*/*Per2* and *Cry1*/*Cry2* genes. PER and CRY proteins are phosphorylated by CK1, and PER/CRY complexes may translocate to the nucleus to inhibit the action of the BC/N heterodimer, thereby inhibiting their own transcription. The *yellow* area depicts the clock input signaling pathways that converge on CREB, which binds to CRE elements in the *Per1* and *Per2* gene promoters and contributes to transcriptional activation, e.g., as a response to a light stimulus received by the retina. *Green* depicts the output pathway of the clock mechanism. BC/N binds to E-boxes in the promoter of a clock-controlled gene (CCG) transmitting time of day information to processes regulated by a CCG. An example of a CCG in the brain is monoamine oxidase A (MAO), which is involved in the degradation of catecholamines such as dopamine. The *brown-shaded* area shows the processes involved in the degradation of PER and CRY. *Purple hexagons* represent substances that influence the kinase and components of the clock mechanisms (*red*)

3 Metabolic Links Between Mood Disorders and the Clock

Mood disorders and their treatment are often associated with an increased risk of metabolic disorders, eating disorders, and obesity (McIntyre 2009). Interestingly, the *Clock*Δ19 mutant mice display in addition to the mania-like behavior also metabolic syndrome (Turek et al. 2005), and hence, a relationship between metabolism, mood, and the clock is apparent in this animal model. The peptides that regulate appetite and circulate in the bloodstream such as ghrelin, leptin, and orexin are altered in their expression in *Clock*Δ19 mutant mice (Turek et al. 2005). These peptides are produced in peripheral organs (ghrelin in the stomach, leptin in white adipose tissue) and bind to their receptors that are expressed in various areas of the brain including areas which are important in mood regulation such as the VTA. Therefore, feeding which affects the production and/or secretion of those peptides plays a role in the regulation of the reward system and hence in mood regulation.

Energy uptake and expenditure also impact on the circadian clock mechanism. Binding of the BMAL1/CLOCK or BMAL1/NPAS2 heterodimer to their cognate E-box sequence in clock gene or clock-controlled gene (CCG) promoters (Fig. 2) is sensitive to the $NAD(P)^+/NAD(P)H$ ratio (Rutter et al. 2001) that is determined by metabolic status. Because nicotinamide phosphoribosyltransferase (NAMPT, the rate-limiting enzyme in the NAD^+ salvage pathway) is transcriptionally regulated by the circadian clock, NAD^+ levels oscillate in the cytosol and probably also in the nucleus in a daily fashion (Nakahata et al. 2009; Ramsey et al. 2009). Disruption of the NAD^+ oscillation by mutating the NAD^+ hydrolase CD38 altered behavioral and metabolic circadian rhythms (Sahar et al. 2011). CD38 deficient mice showed a shortened circadian period and alterations in plasma amino acid levels. This may contribute to abnormal brain function, because many amino acids including tryptophan, tyrosine, and glutamate are precursors of neurotransmitters or are neurotransmitters, respectively.

Nuclear receptors regulate various aspects of metabolism affecting various tissues including the brain. Many nuclear receptors display circadian mRNA expression patterns including REV-ERB (NR1D), ROR (NR1F), and PPAR (NR1C) (Yang et al. 2006). Some of them like the REV-ERBs and RORs are directly involved in the circadian clock mechanism (Fig. 2). A number of nuclear receptors have the potential to interact with the clock component PER2 (Schmutz et al. 2010) linking the clock with metabolism at the posttranslational level.

These observations reinforce the relationship between metabolism, circadian clock, and brain function. Therefore it is tempting to speculate that abnormal metabolism induced by improper eating habits and/or improper sleeping behavior may contribute to the development of mood disorders. This may occur indirectly via alteration of amino acid metabolism and/or synthesis and release of appetite-regulating peptides such as ghrelin and leptin.

4 Treatment of Mood Disorders Changing the Circadian Cycle

Sleep deprivation (SD), bright light therapy, and pharmacological treatments have been successfully used to attenuate depression [reviewed in McClung (2007)]. SD improves depressive symptoms in 40–60 % of patients (Wirz-Justice and Van den Hoofdakker 1999) probably via activation of limbic dopaminergic pathways (Ebert and Berger 1998) and shifting clock phase. In rodents SD decreases immobility in the forced swim test (Lopez-Rodriguez et al. 2004) and stimulates hippocampal neurogenesis (Grassi Zucconi et al. 2006), which is similar to the actions of antidepressant drugs. Furthermore, SD affects phase shifts of the clock in rodents (Challet et al. 2001).

Bright light therapy appears to be effective for several mood disorders including depression (Terman and Terman 2005). Its efficiency is probably rooted in the ability of light to advance clock phase. Similarly to antidepressant drug treatment, it generally takes 2–4 weeks until beneficial effects on mood are seen. Interestingly, selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine produce phase advances in firing of SCN neurons in rat slice cultures (Ehlen et al. 2001; Sprouse et al. 2006). Similarly, agomelatine, which is a melatonin receptor agonist and antagonist of some serotonin receptor isoforms, can cause phase advances in both mice and hamsters (Van Reeth et al. 1997). Long-term antidepressant responses can be induced in bipolar patients applying a combination of SD, morning bright light therapy, and sleep phase advances as a replacement of pharmacological treatment (Wu et al. 2009). Taken together, it appears that phase advancing circadian clock phase elicits antidepressant effects that may involve modulation of SCN activity as well as the serotonergic and melatonin systems.

The mood stabilizer lithium is commonly used for treatment of depressive patients and lengthens the circadian period (Johnsson et al. 1983; Hafen and Wollnik 1994), likely involving the inhibition of GSK3β, which phosphorylates the molecular components PER2 and REV-ERBα of the circadian clock (Iitaka et al. 2005; Yin et al. 2006) (Fig. 2). It produces strong phase delays in circadian rhythms in a variety of organisms, including humans (Atkinson et al. 1975; Johnsson et al. 1983; Klemfuss 1992) and impacts on amplitude and period of the molecular circadian clockwork (Li et al. 2012). Since the strongest effects of lithium are as an antimanic agent, it is interesting that it is acting in an opposite way on circadian period compared to antidepressant treatments (see above).

Other kinases besides GSK3β that may serve as a pharmacological entry points to alter the circadian clock are the casein kinases 1ε and δ (CK1ε/δ). Application of a CKIδ inhibitor (PF-670462) (Fig. 2) to wild-type mice lengthened circadian period accompanied by nuclear retention of the clock protein PER2 (Meng et al. 2010). Interestingly, selective inhibition of CK1ε by PF-4800567 minimally alters circadian clock period (Walton et al. 2009). However, whether these compounds affect mood-related behavior remains to be investigated. Recently, longdaysin, a molecule that targets three kinases, CKIα, CKIδ, and ERK2 was discovered in a large-scale chemical screen (Hirota et al. 2010) (Fig. 2). CKIα inhibition by

longdaysin reduced PER1 phosphorylation and its subsequent degradation. As a consequence, the period in human cells became longer than normal. In vivo, zebra fish embryos displayed a longer clock period after longdaysin administration illustrating the potential of longdaysin to manipulate the circadian clock (Hirota et al. 2010).

Another way of pharmacologically targeting the circadian clock is delivery of substances that activate or inhibit the nuclear receptors of the ROR (NR1F) and REV-ERB (NR1D) families (Fig. 2). Heme seems to be an important ligand influencing REV-ERB transcriptional potential (Yin et al. 2007), and the synthetic agonist GSK4112 (SR6452) (Grant et al. 2010) can compete with heme allowing to start to decipher REV-ERB function. Because REV-ERBs play an important role in adipogenesis, application of heme and GSK4112 (SR6452) has been tested in the regulation of this process. It appears that they are effective modulators of adipogenesis and hence may be useful in the treatment of metabolic disease (Kumar et al. 2010). To which extent the circadian clock is affected by GSK4112 and how mood-related behavior is modulated remain to be tested, although this may be difficult since GSK4112 exhibits no plasma exposure (Kojetin et al. 2011). Recently, a synthetic antagonist for the REV-ERB nuclear receptors was identified (Kojetin et al. 2011), and two REV-ERB agonists with in vivo activity were described which display good plasma exposure (Solt et al. 2012). Administration of these two agonists (SR-9011 and SR9009) altered circadian behavior and clock gene expression in the hypothalamus as well as in the liver, skeletal muscle, and adipose tissue of mice. This resulted in increased energy expenditure. Treatment with these two agonists decreased obesity by reduction of fat mass in diet-induced obese mice, improving dyslipidemia and hyperglycemia (Solt et al. 2012). Hence, it appears that synthetic agonists for REV-ERB may be beneficial in the treatment of sleep and metabolic disorders. Synthetic molecules that bind to the ROR family members have also been identified. SR1078 is an agonist for RORα and RORγ (Wang et al. 2010), whereas SR3335 (ML-176) appears to be a RORα selective inverse agonist (Kumar et al. 2011) (Fig. 2). Future experiments will show how useful these molecules will be in the treatment of metabolic and mood disorders and how they modulate circadian clock function.

Recently, small molecule activators of cryptochrome (CRY) were identified (Hirota et al. 2012). KL001, a carbazole derivative, lengthened circadian period in vitro by preventing ubiquitin-dependent degradation of CRY. It appears that KL001 specifically binds to the FAD binding pocket of CRY and stabilizes it in the nucleus. KL001 repressed glucagon-dependent induction of *Pck1* and *G6pc* genes inhibiting glucagon-mediated activation of glucose production, and therefore, this molecule may provide the basis for a therapeutic approach for diabetes. Since CRY proteins have been implicated in mood disorders (see above), KL001 may also be useful in the development of novel drugs to treat neuropsychiatric disorders.

Taken together, the experimental data in humans and mice suggest that there are two major ways in modulating the circadian clock and clock-related physiological processes. First, environmental factors such as light and food uptake can affect the clock in a long-term manner. Changes in the environment will have to be

continuously present to alter the circadian clock and physiology. Second, pharmacological treatment will allow modulation of the circadian clock in a fast way; however, also this type of treatment will need to have some continuity; otherwise, stop-and-go cycles of circadian timing will stress metabolism and brain function in an unhealthy way. Circadian pharmacology has just seen its dawn, and the future will show how promising the newly discovered agents really are.

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Part III
Chronopharmacology and Chronotherapy

Molecular Clocks in Pharmacology

Erik S. Musiek and Garret A. FitzGerald

Abstract Circadian rhythms regulate a vast array of biological processes and play a fundamental role in mammalian physiology. As a result, considerable diurnal variation in the pharmacokinetics, efficacy, and side effect profiles of many therapeutics has been described. This variation has subsequently been tied to diurnal rhythms in absorption, distribution, metabolism, and excretion, as well as in pharmacodynamic variables, such as target expression. More recently, the molecular basis of circadian rhythmicity has been elucidated with the identification of clock genes, which oscillate in a circadian manner in most cells and tissues and regulate transcription of large sets of genes. Ongoing research efforts are beginning to reveal the critical role of circadian clock genes in the regulation of pharmacologic parameters, as well as the reciprocal impact of drugs on circadian clock function. This chapter will review the role of circadian clocks in the pharmacokinetics and pharmacodynamics of drug response and provide several examples of the complex regulation of pharmacologic systems by components of the molecular circadian clock.

Keywords Circadian clock • Pharmacology • Pharmacokinetics • Pharmacodynamics • CLOCK • Bmal1

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1 Introduction

The maintenance of homeostasis is essential for all biological systems and requires rapid adaptation to the surrounding environment. The evolution of circadian rhythms in mammals exemplifies this, as organisms have developed mechanisms for physiologic modulation to match the varying conditions dictated by a 24-h light–dark cycle. An immense body of evidence over the past century has demonstrated that circadian rhythms influence most key physiologic parameters. More recently, the molecular machinery responsible for generating and maintaining circadian rhythms has been described, and it has become clear that these cell autonomous molecular clocks ultimately control organismal circadian rhythmicity, from endocrine function to complex behavior. Because circadian rhythms are so fundamental to mammalian physiology, it stands to reason that circadian physiologic variation would have significant implications for pharmacology. Indeed, many studies have demonstrated that circadian regulation plays an important role in both the pharmacokinetics and pharmacodynamics of many drugs. Cellular processes ranging from drug absorption to target receptor phosphorylation are influenced by the time of day and in many cases directly by the molecular circadian clock. As a result, circadian regulation can have substantial impact on the efficacy and side effect profile of therapeutics and should thus be considered when developing drug dosing regimens, measuring drug levels, and evaluating drug efficacy. The resultant field of chronopharmacology is dedicated to understanding the importance of time of day in pharmacology and to optimizing drug delivery and design based on circadian regulation of pharmacologic parameters. In this chapter, we will briefly describe the molecular basis of the circadian clock, we will review studies demonstrating the impact of circadian rhythms on physiologic and pharmacologic parameters, and we will describe the molecular mechanisms by which the circadian clock influences pharmacologic targets. The goal of this chapter is to provide a framework within which to consider circadian influences on future investigations in pharmacology.

2 Molecular Anatomy of the Mammalian Circadian System

The generation and maintenance of circadian rhythms in mammals depends both on core molecular machinery and on a complex anatomical organization. As a result, circadian rhythmicity requires functional cell autonomous oscillation (Buhr and Takahashi 2013), neuroanatomical circuitry and neurotransmission (Slat et al. 2013), and paracrine and endocrine signaling systems (Kalsbeek and Fliers 2013). Circadian rhythms are maintained via the function of tissue-specific molecular clocks that are synchronized through communication with the master clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus, which is entrained to light by an input from the retina (Reppert and Weaver 2002). The SCN

synchronizes peripheral clocks in various organs to light input via regulation of diverse systems including the autonomic nervous system, the pineal gland, and the hypothalamic–pituitary axis. Nevertheless, isolated peripheral tissues and even cultured cells maintain circadian rhythmicity in the absence of input from the SCN (Baggs et al. 2009). The core molecular clock components responsible for this cell autonomous rhythmicity consist of "positive limb" components, Bmal1 and CLOCK, which are basic helix–loop–helix/PER-arylhydrocarbon receptor nuclear translocator single-minded protein (bHLH/PAS) transcription factors that heterodimerize and bind to E-box motifs in a number of genes, driving transcription (Reppert and Weaver 2002). Another bHLH/PAS transcription factor, NPAS2, which is highly expressed in the forebrain, can alternatively heterodimerize with Bmal1 to facilitate transcription (Reick et al. 2001; Zhou et al. 1997). Bmal1/CLOCK drives transcription of several distinct negative feedback ("negative-limb") components, including two cryptochrome (Cry1,2) genes and three Period genes (Per1–3). Per and Cry proteins then heterodimerize and repress Bmal1/Clock-mediated transcription (Kume et al. 1999). Molecular clock oscillation is also influenced by two other Bmal1/CLOCK targets, RORα (retinoid-related orphan receptor alpha) and REV-ERBα. RORα binds to specific elements and enhances Bmal1 transcription (Akashi and Takumi 2005; Sato et al. 2004). REV-ERBα, another orphan nuclear receptor involved in glucose sensing and metabolism, competes with RORα for DNA binding and suppresses Bmal1 transcription (Preitner et al. 2002). The core clock machinery (referred to herein as the circadian clock) is found in most tissues and has been estimated to mediate the circadian transcription of roughly 10–20 % of active genes (Ptitsyn et al. 2006).

Recently, evidence has been provided that the regulation of the molecular clock periodicity is complex and subject to a wide array of influences. The circadian protein CLOCK has intrinsic histone acetyltransferase activity and can thus participate in epigenetic regulation of chromatin structure and acetylation of other proteins, including molecular clock components (Doi et al. 2006; Etchegaray et al. 2003; Sahar and Sassone-Corsi 2013). Indeed, posttranslational modifications of molecular clock proteins, including phosphorylation, SUMOylation, and acetylation, are critical for tuning of molecular clock function (Cardone et al. 2005; Gallego and Virshup 2007; Lee et al. 2001). Clock function is modified via input from diverse signaling proteins including casein kinase I epsilon (Akashi et al. 2002), the deacetylase SIRT1 (Asher et al. 2008; Belden and Dunlap 2008; Nakahata et al. 2008), the metabolic sensor AMP kinase (Lamia et al. 2009), and the DNA repair protein Poly-ADP ribose polymerase (Asher et al. 2010). Molecular clock function is also sensitive to the redox status of the cell (Rutter et al. 2001) and in turn regulates intracellular NAD+ levels through regulation of the enzyme nicotinamide phosphoribosyltransferase (NAMPT) (Nakahata et al. 2009; Ramsey et al. 2009). Thus, the molecular clock is sensitive to a wide array of physiologic (and pharmacologic) cues.

3 Circadian Regulation of Pharmacokinetics

Circadian systems have been shown to influence drug absorption, distribution, metabolism, and excretion (ADME). Each of these processes plays a role in determining blood levels on a drug. Thus, time of day of drug administration, as well as the synchronization of the peripheral molecular clocks in several key organs (including the gut, liver, and drug target tissue), can have substantial effect on drug levels and bioavailability.

3.1 Absorption

The absorption of orally administered drugs depends on several factors including physiologic parameters of the GI tract (blood flow, pH, gastric emptying) and expression and function of specific uptake and efflux pumps on epithelial cell surfaces. Gastric pH plays an important role in the absorption of drugs, as lipophilic molecules are absorbed less readily under acidic conditions. Since the initial demonstration of circadian variation in gastric pH in humans by Moore et al. in 1970, considerable evidence has accumulated showing the existence of circadian clocks within the gut and the importance of these clocks in the timing of gut physiology (Bron and Furness 2009; Hoogerwerf 2006; Konturek et al. 2011; Moore and Englert 1970; Scheving 2000; Scheving and Russell 2007). The production of the hormone ghrelin by oxyntic cells in the stomach is regulated by circadian clock genes and mediates circadian changes in activity prior to feeding, known as "food anticipatory activity" (LeSauter et al. 2009). Oxyntic cells tune circadian oscillation of the GI tract to food intake patterns rather than light. Other gut parameters which show circadian oscillation include gastric blood flow and motility, both which are increased during daylight hours and decreased at night (Eleftheriadis et al. 1998; Goo et al. 1987; Kumar et al. 1986).

The absorption of many therapeutic agents is highly dependent on the expression of specific transporter proteins in the gut. Many of these transporters show circadian variation in expression, and several have been demonstrated to be directly regulated by the core circadian clock. In mice, the xenobiotic efflux pump Mdr1a (also known as p-glycoprotein) exhibits circadian regulation (Ando et al. 2005) which is controlled by the circadian clock-mediated expression of hepatic leukemia factor (HLF) and E4 promoter binding protein-4 (E4BP4) (Murakami et al. 2008). Several other efflux pumps, including Mct1, Mrp2, Pept1, and Bcrp, also show circadian expression patterns (Stearns et al. 2008). The circadian regulation of both physiologic parameters and the expression of specific proteins involved in drug absorption provide a mechanistic basis for understanding observed time-of-day effects in the absorption of many drugs. Circadian patterns of absorption are most pronounced in lipophilic drugs, with greater absorption occurring during the day than at night (Sukumaran et al. 2010). Interestingly, absorption of the lipophilic beta blocker

propranolol was significantly greater in the morning than at night, while the water-soluble beta blocker atenolol showed no significant diurnal variation in absorption (Shiga et al. 1993). While wild-type mice show diurnal variation in lipid absorption, with greater absorption occurring at night, this diurnal variation was lost in Clock mutant mice. As a result, Clock mutants demonstrated significantly greater lipid absorption in a 24-h period (Pan and Hussain 2009). Several lipid transport proteins, including microsomal transport protein (MTP), are also regulated by the circadian clock in mice, suggesting that intestinal uptake of lipids and lipophilic drugs may be under circadian clock control in humans (Pan and Hussain 2007, 2009; Pan et al. 2010).

As a result of these diurnal variations in physiologic parameters and transporters/efflux pumps, the absorption on many drugs, including diazepam (Nakano et al. 1984), acetaminophen (Kamali et al. 1987), theophylline (Taylor et al. 1983), digoxin (Lemmer 1995), propranolol (Shiga et al. 1993), nitrates (Scheidel and Lemmer 1991), nifedipine (Lemmer et al. 1991), temazepam (Muller et al. 1987), and amitriptyline (Nakano and Hollister 1983), is sensitive to the time of day of administration. The absorption of most drugs is greater in the morning, paralleling morning increases in gut perfusion and gastric pH. Thus, circadian factors must be considered when developing oral therapeutic administration regimens.

3.2 Distribution

The volume of distribution of a given drug is determined largely by that drug's lipophilicity and plasma protein binding affinity, as well as the abundance of plasma proteins. Circadian regulation of the concentration of plasma proteins can thus theoretically induce circadian changes in the volume of distribution of a drug. Circadian regulation of plasma levels of several proteins which commonly bind drugs has been reported (Scheving et al. 1968). The degree of protein binding of several drugs, including the antiepileptic agents, valproic acid and carbamazepine, and the chemotherapeutic cisplatin, varies in a diurnal manner which correlates appropriately with changes in plasma albumin level (Hecquet et al. 1985; Patel et al. 1982; Riva et al. 1984). Variations in the free (active) fraction of drug have important implications for both the efficacy and side effect profile of these drugs. Circadian variation in the levels and saturation of the glucocorticoid-binding protein transcortin has also been described, which may influence the efficacy of exogenously administered corticosteroids (Angeli et al. 1978). As plasma protein levels influence the distribution of a wide array of drugs beyond those described here, it is likely that circadian regulation of these proteins has a significant impact on pharmacology.

The ability of a drug to cross membranes between different tissue compartments is also a determinant of drug distribution. Because many water-soluble agents require the expression of certain membrane-bound proteins (transporters, channels) to transit between tissue compartments and reach their receptors, the circadian

regulation of such transporter has implications for drug distribution. As described above in the section on absorption, a variety of drug transporters which are critical for drug distribution in tissues are regulated by circadian mechanisms (Ando et al. 2005; Stearns et al. 2008).

3.3 Metabolism

Hepatic metabolism of drugs generally occurs in two phases which are carried out by distinct set of enzymes. Phase I metabolism usually involves oxidation, reduction, hydrolysis, or cyclization reactions, and is often carried out by the cytochrome P450 family of monoxidases. Phase II metabolism involves conjugation reactions catalyzed by glutathione transferases, UDP glucuronyl-, methyl-, acetyl-, and sulfotransferases, leading to the production of polar conjugates which can be easily excreted. There is an evidence of circadian regulation of both phases of drug metabolism.

Diurnal variation in the levels and activity of various phase I metabolic enzymes in the liver of rodents has been long appreciated (Nair and Casper 1969). Experiments in mice and rats have demonstrated that many cytochrome P450 (CYP) genes show a circadian expression profile (Desai et al. 2004; Hirao et al. 2006; Zhang et al. 2009). Several non-CYP phase I enzymes also show diurnal variation. Ample evidence has accumulated which shows that phase I metabolic enzyme expression is regulated by the circadian clock machinery (Panda et al. 2002). The core circadian clock exerts transcriptional regulation indirectly through circadian expression of the PAR bZIP transcription factors DBP, HLF, and TEF, which in turn regulate expression of target genes. In mice, the expression of Cyp2a4 and Cyp2a5 demonstrated robust circadian oscillation and was shown to be directly controlled by the circadian clock output protein DBP (Lavery et al. 1999). In mice with targeted deletion of all three PAR bZIP proteins, severe impairment in hepatic metabolism was observed as well as downregulation of the phase I enzymes Cyp2b, 2c, 3a, 4a, and CYP oxidoreductase (Gachon et al. 2006). These mice also had diminished expression of a diverse array of phase II enzymes including members of the glutathione transferase, sulfotransferase, aldehyde dehydrogenase, and UDP-glucuronosyltransferase families. Similarly, microarray analysis of gene expression for the livers of mice with deletion of the circadian genes RORα and -γ revealed marked downregulation of numerous phase I and II metabolic enzymes (Kang et al. 2007). Thus, circadian transcriptional regulation of phase I genes has major implications for drug metabolism.

Phase II metabolism is also regulated by circadian mechanisms. Initial studies in mice demonstrated diurnal variation in hepatic glutathione-S-transferase (GST) activity, with greatest activity being present during the dark (active) phase (Davies et al. 1983). However, subsequent studies also observed circadian regulation of GST activity, but with the acrophase during the light (rest) period (Inoue et al. 1999; Jaeschke and Wendel 1985; Zhang et al. 2009). Diurnal variation in

UDP-glucuronosyltransferase and sulfotransferase activities has also been described, which appeared to be dependent on feeding cues (Belanger et al. 1985). As mentioned previously, genetic deletion of the circadian output genes DBP, HLF, and TEF, or the circadian regulators RORα and -γ, caused large-scale disruption of phase II enzyme expression in liver, suggesting a prominent role for the circadian clock in phase II enzyme regulation. The expression the aryl hydrocarbon receptor (AhrR), a transcription factor which mediates toxin-induced phase II enzyme induction, is also regulated by the circadian clock. Several studies have demonstrated that AhR is under transcriptional regulation of the core circadian clock and that AhR-mediated induction of Cyp1a1 by the AhR agonist benzo[a] pyrene is highly dependent on time of day of administration (Qu et al. 2010; Shimba and Watabe 2009; Tanimura et al. 2011; Xu et al. 2010). Circadian regulation of hepatic blood flow has been suggested to regulate drug metabolism, particularly for drugs with a high extraction rate (Sukumaran et al. 2010).

3.4 Excretion

Urinary excretion of metabolized drugs is highly dependent on factors related to kidney function. As diurnal variation in renal parameters including glomerular filtration rate, renal plasma flow, and urine output have been described, it is not surprising that diurnal variation in the urinary excretion of several drugs has been observed (Cao et al. 2005; Gachon et al. 2006; Minors et al. 1988; Stow and Gumz 2010). In mice, the circadian clock regulates the expression of several renal channels and transporter proteins, including epithelial sodium transporters, suggesting a possible direct role for clock genes in drug excretion (Gumz et al. 2009; Zuber et al. 2009). Circadian regulation of urinary pH could also contribute to variations in drug excretion, as many drugs become protonated at high pH which enhances excretion. Urinary pH shows diurnal variation in humans, perhaps explaining the diurnal variation in the excretion of certain drugs such as amphetamine (Wilkinson and Beckett 1968).

4 Circadian Regulation of Pharmacodynamics

Circadian mechanisms regulate many factors which influence the efficacy of drugs aside from their metabolism. Rhythmic alterations in the expression of target receptors, transporters and enzymes, intracellular signaling systems, and gene transcription all have been reported and have the potential to impact the efficacy of therapeutics. While an extensive literature has emerged which examines the effect of various drugs on the phase and rhythmicity of circadian clocks, there has been less emphasis on the effect of circadian clocks on drug targets. In the past, this work was largely limited to the description of diurnal changes in the levels of

various receptors, enzymes, and metabolites, which suggested but could not prove circadian clock involvement. However, the recent development of an array of mouse genetic models with deletion or disruption of specific circadian clock genes has led to some initial discoveries demonstrating the pivotal role of the molecular clock in target function and drug efficacy. The chronopharmacology literature is extensive and often descriptive, and an exhaustive account of the circadian regulation of all areas of pharmacology is beyond the scope of this chapter. Instead, illustrative examples from several areas of pharmacology will be presented. Circadian mechanisms play critical roles in cancer and chemotherapeutics, but because this topic is reviewed elsewhere in this volume (Ortiz-Tudela et al. 2013), it will not be discussed herein. Similarly, the critical role of circadian clocks in cardiovascular pharmacology has been reviewed extensively elsewhere (Paschos et al. 2010; Paschos and FitzGerald 2010) and is not discussed.

4.1 Circadian Clocks and Neuropharmacology

The regulation of neurotransmitter signaling in the central nervous system is highly complex and is the ultimate target of hundreds of drugs designed to treat a wide variety of disorders, from depression to Parkinson's disease. Ligand-binding studies performed on mouse and rat brain homogenates have demonstrated time-of-day variation in the binding affinity of several neurotransmitter receptor families, suggesting possible circadian regulation of neurotransmitter signaling (Wirz-Justice 1987). Indeed, diurnal variation in radioligand binding which persists in constant darkness has been reported for α- and β-adrenergic, GABAergic, serotonergic, cholinergic, dopaminergic, and opiate receptors (Cai et al. 2010; Wirz-Justice 1987). The regulation of several enzymes involved in the catabolism of neurotransmitters also shows circadian variation in the brain (Perry et al. 1977a, b). As an example, the levels of monoamine oxidase A (MAO-A), which metabolizes catecholamines and serotonin and is a target of MAO inhibitor antidepressant drugs, are regulated by the core circadian clock (Hampp et al. 2008). Importantly, several of these same neurotransmitter systems, including serotonergic, cholinergic, and dopaminergic nuclei, also play critical roles in tuning the circadian clock. Thus, a bidirectional relationship between neurotransmitter regulation and circadian clock function exists in the brain (Uz et al. 2005; Yujnovsky et al. 2006).

Serotonin represents a particularly robust example of the bidirectional relationships between drugs and the circadian clock. Serotonin is a neurotransmitter which mediates a wide variety of effects in the central nervous system, but is perhaps most studied from a pharmacologic standpoint for its role in depression. Levels of serotonin show circadian rhythmicity in several brain regions, including the SCN, pineal gland, and striatum, which peaks at the light/dark transition and persists in constant darkness (Dixit and Buckley 1967; Dudley et al. 1998; Glass et al. 2003; Snyder et al. 1965). One reason for this is the fact that serotonin is converted to melatonin in the pineal gland during the dark phase by action of the

enzyme serotonin *N*-acetyltransferase, which is expressed in a circadian manner (Bernard et al. 1997; Deguchi 1975). Circadian regulation of serotonin is dependent on input from the sympathetic nervous system, as adrenergic blockade or ablation of the superior cervical ganglion abrogated this diurnal rhythm (Snyder et al. 1965, 1967; Sun et al. 2002). Diurnal variation in the serotonin transporter, the major target of selective serotonin reuptake inhibitors (SSRIs, the major class of antidepressant drugs), has been described in female rats, but no data exists for humans (Krajnak et al. 2003). A wide variety of antidepressant, anxiolytic, atypical antipsychotic, and antiemetic drugs target serotonin, either by increasing synaptic serotonin via inhibition of reuptake transporters or by agonism or antagonism of specific serotonin receptors. Thus, the circadian regulation of serotonin levels has implications for the dosing of these classes of drugs. Conversely, considerable evidence has accumulated in a variety of species showing that serotonin also plays a key role in regulating the circadian clock, as serotonergic signaling is required for normal SCN rhythmicity (Edgar et al. 1997; Glass et al. 2003; Horikawa et al. 2000; Yuan et al. 2005). Accordingly, drugs which modulate serotonin signaling have pronounced effects on circadian clock function. As an example, the selective serotonin reuptake inhibitor (SSRI) fluoxetine induces marked phase advances in SCN rhythms in mice (Sprouse et al. 2006). In a more global example, Golder et al. detected circadian rhythms in mood by analyzing millions of messages on the social networking website Twitter (Golder and Macy 2011). Mood peaked in the morning and declined as the day continued and was consistent across diverse cultures. Thus, considerable circadian complexity must be considered when designing therapeutic strategies which target serotonergic systems.

4.2 Circadian Clocks in Metabolic Diseases

Recent studies in genetically modified mice have revealed critical roles for circadian clock genes in metabolic diseases such as diabetes and obesity. Circadian clock genes regulate key metabolic processes such as insulin secretion, gluconeogenesis, and fatty acid metabolism (Bass and Takahashi 2010). A dominant negative mutation of CLOCK in mice results in obesity, hyperlipidemia, and diabetes (Marcheva et al. 2010; Turek et al. 2005; for a review, see Marcheva et al. 2013). Bmal1/CLOCK heterodimers directly enhance transcription at the peroxisome proliferator response element, thereby contributing to lipid homeostasis (Inoue et al. 2005). Furthermore, expression of the nuclear hormone receptor peroxisome proliferator-activated receptor alpha (PPAR-α), the pharmacologic target of the fibrate drugs, follows a diurnal pattern in the liver which is abrogated in CLOCK mutant mice (Lemberger et al. 1996; Oishi et al. 2005). PPAR-γ, which is a major target of several antidiabetic drugs including the thiazolinediones, is also under circadian transcriptional control of the clock-mediated PAR bZIP transcription factor E4BP4 (Takahashi et al. 2010). Much like the serotonin system, PPAR-α

and -γ also regulate the expression and function of circadian clock genes in a reciprocal manner (Canaple et al. 2006; Wang et al. 2008). The critical role of core clock genes in the control of metabolism was further reinforced by the finding that treatment of mice with synthetic small molecule agonists of REV-ERBα/β caused large-scale alterations in metabolism and enhanced energy expenditure, reducing obesity, hyperlipidemia, and hyperglycemia in mice fed a high-fat diet (Solt et al. 2012). Conversely, mice lacking both REV-ERBα and β developed dyslipidemia (Cho et al. 2012). Interestingly, a recent report demonstrated that the negative-limb circadian clock gene cryptochrome 1 (Cry1) blocks glucagon-mediated gluconeogenesis in mice during the dark phase (Zhang et al. 2011). The proposed mechanism of gluconeogenesis suppression by Cry1 was through suppression of G-protein coupled receptor (GPCR)-induced cAMP production. Inhibition of gluconeogenesis was also observed in hepatocytes treated with a novel small molecule cryptochrome-stabilizing agent (Hirota et al. 2012). As cryptochrome genes are expressed in most tissues in a circadian manner as part of the core clock machinery, these findings have broad implications not only for metabolic disease therapy but also for understanding the role of the circadian clock in the regulation of GPCR signaling in general (Zhang et al. 2011). As GPCRs represent the most common therapeutic targets in pharmacology, it appears likely that the influence of circadian mechanisms on pharmacodynamics is just beginning to be appreciated. Another emerging mechanism for the regulation of receptor signaling is acetylation by molecular clock components. CLOCK has intrinsic acetyltransferase activity and can acetylate histones and other proteins (Curtis et al. 2004; Doi et al. 2006). Recently, it has been demonstrated that CLOCK acetylates the glucocorticoid receptor (GR), a nuclear receptor which is the target for exogenous glucocorticoids used to treat a wide variety of inflammatory diseases (Kino and Chrousos 2011a, b; Nader et al. 2009). CLOCK acetylates GR in a circadian manner, suppressing its activity and decreasing tissue sensitivity to glucocorticoids (Charmandari et al. 2011). Cry1 and Cry2 also regulate the function of the glucocorticoid receptor, strongly suppressing the transcriptional response to glucocorticoids in the liver by associating with GR-responsive genomic elements in a ligand-dependent manner and suppressing GR signaling (Lamia et al. 2011). These findings have broad implications for understanding endogenous cortisol regulation and the pharmacology of exogenous glucocorticoids in the treatment of disease and may serve as a model for the regulation of other receptors by the circadian clock.

4.3 Aging, Clocks, and Pharmacology

Certain circadian rhythms, such as hormonal rhythms and sleep cycles, phase shift and then decline with age across species (Harper et al. 2005). In Drosophila, the function of the molecular clock is highly sensitive to oxidative stress, and dysfunction of the molecular clock is exacerbated by aging (Koh et al. 2006;

Zheng et al. 2007). In mice and humans, expression of molecular clock genes declines and becomes dysregulated with age (Cermakian et al. 2011; Kolker et al. 2004; Nakamura et al. 2011; Weinert et al. 2001). Furthermore, deletion of Bmal1 or mutation of Clock in mice results in an accelerated aging phenotype, suggesting a bidirectional role of clock genes in aging (Antoch et al. 2008; Kondratov et al. 2006). The interaction between aging and circadian systems has several important implications for pharmacology. First, because circadian mechanisms influence nearly every aspect of pharmacology, the disruption of normal circadian function in elderly patients (as well as in shift workers, patients with chronic sleep disturbances, and others) is likely to have significant impact on drug efficacy and tolerance, and must be considered. Second, the impact of certain drugs on circadian clock function should also be considered in aged populations, as these patients are already likely to have some degree of clock dysfunction and may thus be more susceptible to drug-induced alteration in circadian rhythmicity. Finally, the circadian clock itself may become a therapeutic target for the amelioration of age-related diseases. Indeed, several studies have already demonstrated the feasibility of developing "clock drugs" which alter clock gene expression and rhythms (Hirota et al. 2008, 2010, 2012).

5 Conclusions

Circadian biology influences nearly every aspect of physiology and pharmacology. Ongoing research has begun to unveil the molecular mechanisms by which circadian clock genes regulate pharmacokinetic and pharmacodynamic processes. It is also becoming readily apparent that drugs can influence the rhythmicity of circadian clocks and can potentially alter physiology, perhaps in some case with unintended consequences. Ongoing investigation into novel mechanisms by which molecular clocks alter pharmacologic parameters, the consequences of these alterations on drug efficacy and tolerability, and possible methods to use circadian biology to our pharmacologic advantage is needed. At this point, it is clear that circadian regulation must be considered when designing and dosing drugs, particularly when therapeutic studies do not provide the expected results.

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Cancer Chronotherapeutics: Experimental, Theoretical, and Clinical Aspects

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Abstract The circadian timing system controls cell cycle, apoptosis, drug bioactivation, and transport and detoxification mechanisms in healthy tissues. As a consequence, the tolerability of cancer chemotherapy varies up to several folds as a function of circadian timing of drug administration in experimental models. Best antitumor efficacy of single-agent or combination chemotherapy usually corresponds to the delivery of anticancer drugs near their respective times of best tolerability. Mathematical models reveal that such coincidence between chronotolerance and chronoefficacy is best explained by differences in the circadian and cell cycle dynamics of host and cancer cells, especially with regard circadian entrainment and cell cycle variability. In the clinic, a large improvement in tolerability was shown in international randomized trials where cancer patients received the same sinusoidal chronotherapy schedule over 24 h as compared to constant-rate infusion or wrongly timed chronotherapy. However, sex, genetic background, and

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lifestyle were found to influence optimal chronotherapy scheduling. These findings support systems biology approaches to cancer chronotherapeutics. They involve the systematic experimental mapping and modeling of chronopharmacology pathways in synchronized cell cultures and their adjustment to mouse models of both sexes and distinct genetic background, as recently shown for irinotecan. Model-based personalized circadian drug delivery aims at jointly improving tolerability and efficacy of anticancer drugs based on the circadian timing system of individual patients, using dedicated circadian biomarker and drug delivery technologies.

Keywords Cancer • Circadian rhythms • Chronotherapy • Survival • Chronotolerance • Chronoefficacy • Mathematical models • Clinical trials

1 Context

Cancer is a systemic disease, and therefore, it can profoundly affect daily activities, sleep, and feeding, as well as cellular metabolism (Mormont and Lévi 1997; Barsevick et al. 2010). Thus, cancer patients often experience fatigue, which prevents them to carry on their daily routines (Weis 2011). Cancer patients on chemotherapy further experience treatment-related adverse events such as nausea, vomiting, or diarrhea, which also impair their quality of life (Van Ryckeghem and Van Belle 2010). Besides, most anticancer treatments are administered within hospital wards, a condition which also disrupts the daily routines of cancer patients. Indeed, cancer, treatments and hospitalization can alter the rest–activity pattern of patients.

The endogenous circadian rhythm in rest–activity is controlled by the suprachiasmatic nuclei in the hypothalamus (Hastings et al. 2003). This rhythm has been commonly evaluated in cancer patients as a biomarker that reflects the robustness of the circadian timing system (CTS) (Mormont et al. 2000; Ancoli-Israel et al. 2003; Calogiuri et al. 2011; Berger et al. 2007). Moreover, patients suffering from circadian disruption have a poorer survival outcome, compared to those with a robust CTS, as indicated with rest–activity or cortisol patterns (Mormont et al. 2000; Sephton et al. 2000; Innominato et al. 2009). Studies in mice have backed up the above clinical findings, since anatomical or functional SCN suppression or clock gene mutations accelerated cancer progression (Filipski et al. 2002, 2004, 2005, 2006; Fu et al. 2002; Otálora et al. 2008).

On the other hand, treatment effects vary according to dosing time. This has especially been shown both for the tolerability and the efficacy of anticancer drugs. The findings have led to the concept of cancer chronotherapy, with circadian timing of drug delivery playing a crucial role for improving tolerability and/or efficacy (Lévi et al. 2010). Cancer chronotherapeutics is a field of research that aims at optimizing cancer treatments through the integration of circadian clocks in the design of anticancer drug delivery (Lévi and Okyar 2011).

2 Circadian-Based Cancer Treatments

The CTS rhythmically controls both drug metabolism and cellular detoxification, thus alters drug interactions with their molecular targets as well as DNA repair and apoptosis over 24 h in healthy tissues. The CTS also regulates healthy cell cycle (Antoch and Kondratov 2013). Since many anticancer drugs target a given stage of the cell division cycle, the clock-controlled cell proliferation events also represent a critical determinant of anticancer drug cytotoxicity (Haus 2002; Granda et al. 2005; Tampellini et al. 1998; Smaaland et al. 2002). Both orders of mechanisms are responsible for large and predictable changes in the tolerability of anticancer drugs. In contrast, cell divisions usually occur in an asynchronous fashion in cancer tissues (Fu and Lee 2003; Lévi et al. 2007a). The temporal dissociation between healthy and cancer tissues provides the main rationale of cancer chronotherapy, which aims at minimizing treatment toxicities, while maximizing efficacy through properly timing treatment delivery (Lévi and Okyar 2011). However, there may be a circadian regulation of malignant tumors that can involve the CTS control of vascular endothelial growth factor-mediated neo-angiogenesis (Koyanagi et al. 2003; Lévi et al. 2010).

Tolerability rhythms have been demonstrated for more than 40 anticancer drugs, including cytokines, cytostatics, antiangiogenic agents, and cell cycle inhibitors in mice or rats synchronized with an alternation of 12 h of light and 12 h of darkness (Lévi et al. 2010). Lethal toxicity and/or body weight loss following anticancer drug administration usually varies two- to tenfold as a function of circadian timing (Lévi and Schibler 2007). Experimental evidence reveals that both dose and circadian timing jointly play a critical role for the antitumor efficacy of 28 anticancer agents in mice, using tumor growth inhibition or increase in life span as established measures of treatment efficacy in experimental systems (Lévi et al. 2010).

2.1 Circadian Control of Detoxification

Chronotolerance and chronoefficacy result from an array of cellular rhythms involving drug detoxification and/or bioactivation enzymes as well as drug transporters. These cellular rhythms can now be explored in synchronized cell cultures (Lévi et al. 2010; Ballesta et al. 2011; Dulong et al. Chronopharmacology of irinotecan at cellular level. Unpublished). They translate into the well-known circadian changes shown for drug exposure and elimination at whole organism level. In mice, circadian clocks control Phase I metabolism enzymes such as CYP450 and carboxylesterases as well as Phase II detoxification enzymes such as glucuronosyltransferases and glutathione S-transferases enzymes (Martin et al. 2003) and ABC transporters including abcb1a/b and abcc2 (Murakami et al. 2008; Okyar et al. 2011).

2.2 Circadian Control of Cell Cycle

Each cell has a molecular clock within it consisting of a set of feedback loops that create oscillations in gene expression at mRNA and protein levels with a period of about 24 h (Ko and Takahashi 2006; Huang et al. 2011; for a review see Buhr and Takahashi 2013).

These clock genes control the rhythmic expression of up to 10 % of the transcriptome (Panda et al. 2002; Storch et al. 2002). Besides, some posttranslational rhythms appear to be independent from the transcriptional rhythms (O'Neill et al. 2011; for a review, see O'Neill et al. 2013). Additionally, nongenetic circadian clocks have recently been described in red blood cells (O'Neill and Reddy 2011). Neither the mechanistic links between these different circadian oscillators nor their respective relevance for cancer chronotherapy is currently known.

Clock genes participate in several physiological processes in cells, including the regulation of cell cycle (Fig. 1; see also Antoch and Kondratov 2013). For instance, the dimer CLOCK–BMAL1 activates the expression of *cMyc* and *p21*, whose product proteins play an important role on proliferation and apoptosis (Khapre et al. 2010). Furthermore, CLOCK:BMAL1 participates also on the activation of *p53*, a proapoptotic gene, and *Wee1*, whose protein prevents the transition from G2 to mitosis by the inactivating phosphorylation of the complex CDC2/CyclinB1 (Hunt et al. 2007). The clock machinery further regulates apoptosis through the rhythmic expression of proapoptotic (*Bax*) and antiapoptotic (*Bcl2*) genes (Granda et al. 2005). P53 protein plays an important role in tumor suppression, through promoting apoptosis in healthy cells exposed to DNA-damaging agent or initiating oncogenic transformation. In the absence of p53, p73 is able to substitute p53 as tumor suppressor. Thus, apoptosis was increased, as a result of the enhanced induction of *p73* in cancer cells with both clock and P53 silencing ($Cry1^{-/-}$ $Cry2^{-/-}$ $p53^{-/-}$). This finding suggests a possible therapeutic role for cryptochrome silencing in those cancer cells with P53 mutation, which usually display a most aggressive malignant phenotype (Lee and Sancar 2011). The functional status of the CLOCK:BMAL1 heterodimer was shown to alter chronotolerance for chemotherapy in wild-type mice. Conversely, mice with circadian clock mutation $Clock^{\Delta 19/\Delta 19}$ or $Bmal1^{-/-}$ displayed severe toxicity of the alkylating agent cyclophosphamide irrespective of dosing time, while $Cry1^{-/-}$ and $Cry2^{-/-}$ mice displayed improved yet time-invariant tolerability for this drug as compared to wild-type mice (Gorbacheva et al. 2005).

Both DNA damage sensing and DNA repair are controlled in part by the rhythmic expression of XPA (Kang et al. 2010). Core circadian genes seem to respond directly to radiation, so that the disruption of *Per2* prevents the response of all core circadian genes to radiation (Fu and Lee 2003). Such clock effects of radiation are in line with the demonstration that ionizing radiation produces circadian phase shifts in dose- and time-dependent manner (Oklejewicz et al. 2008). Thus, genotoxic stress can modulate the molecular clock, a critically relevant finding for cancer chronotherapy involving DNA-damaging drugs (Miyamoto et al. 2008).

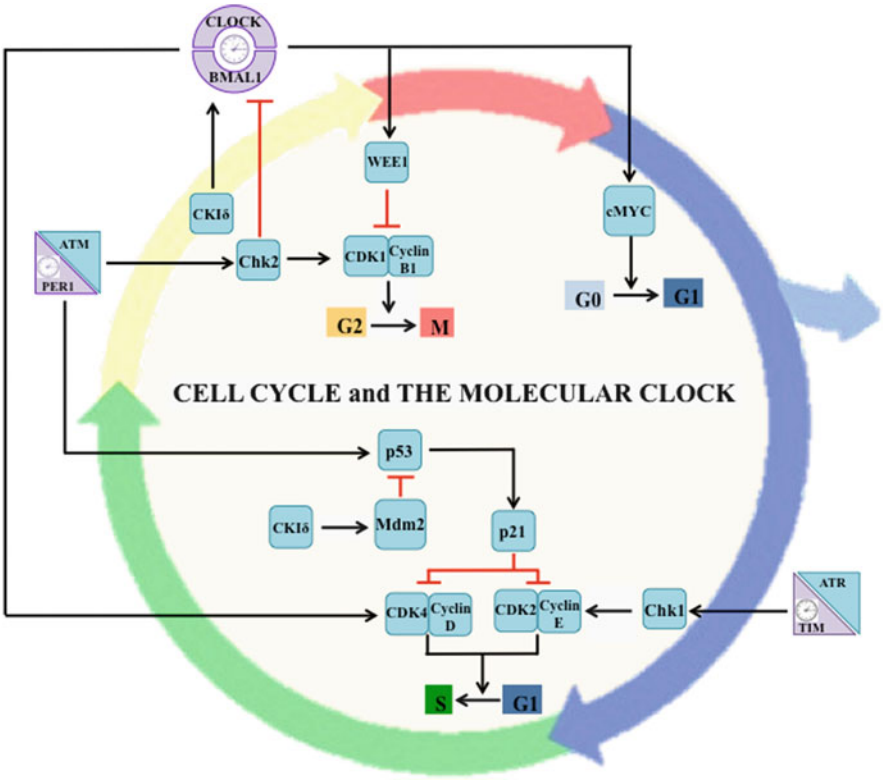

Fig. 1 Hypothetical scheme describing the interactions between the molecular clock and the cell cycle. The 24-h rhythmic oscillation generated by the molecular clock is produced by interwoven feedback loops involving at least 15 clock genes and proteins. PER and CRY proteins form heterodimers that interfere with the CLOCK:: BMAL1 heterodimer which activates the mRNA transcription of *Per, Cry, Rev-erb,* and *Dec* genes. Subsequently, REV-ERBα protein blocks *Bmal1* transcription, which is activated by RORα protein (not shown). The CLOCK::BMAL1 heterodimer also directly controls the transcriptional activity of clock-controlled genes such as *Wee1*, *cMyc, Ccnd1*, and *P21* which regulate the cell division cycle. In addition, PER1 protein binds to ATM (ataxia telangiectasia mutated). Both PER1 and ATM can phosphorylate P53 and CHK2. P53 both regulates apoptosis and arrests the cell cycle in G1 phase through activating *P21* transcription, among many other functions. P21 inhibits the complexes formed by CCNE and CCND thus preventing cell cycle progression from S to G2 phase. CHK2 (cell cycle checkpoint kinase 2) protein can both prevent the cell cycle control by the CLOCK:: BMAL1 dimer and activate the CCNB1–CDK1 complex that is required for the cycling cell to enter mitosis (M-phase)

2.3 Clock Genes and Cancer

Both the expression of clock genes and their circadian pattern are usually disrupted in most experimental tumors growing in mice, especially following the initial latency phase (Filipski et al. 2005; Li et al. 2010). Cancer progression was reportedly counteracted by *Per* genes expression. Thus, the overexpression of *Per1*

inhibited growth in human cancer cell lines and increased apoptosis after ionizing radiation. In contrast, *Per1* silencing prevented radiation-induced apoptosis (Gery et al. 2006). The downregulation of clock gene *Per2* was also associated with increased cell proliferation, while its overexpression promoted apoptosis (Fu and Lee 2003; Gery et al. 2005; Wood et al. 2008). These and other experimental findings are in line with the mRNA or protein downregulation of *Per1* or *Per2* in several human cancers (Gery et al. 2006; Chen et al. 2005; Yeh et al. 2005; Innominato et al. 2010). Indeed clock genes alterations in tumors and/or in hosts have been reported to respectively affect patient survival and cancer risk (Table 1). Thus, polymorphisms in circadian genes have been associated with cancer risk and patient survival for non-Hodgkin's lymphoma (Hoffman et al. 2009; Zhu et al. 2007), prostate cancer (Chu et al. 2008), or breast cancer (Yi et al. 2010). For example, a single-nucleotide polymorphism (SNP) in NPAS2 confers a 49 % decrease in breast cancer risk (Zhu et al. 2008), while *Cry2* polymorphisms are also associated with an increased risk of non-Hodgkin's lymphoma and prostate cancer (Chu et al. 2008; Hoffman et al. 2009).

3 Clinical Options in Cancer Chronotherapy

Conventional cancer therapies involve the timing of drugs according to hospital routine and staff working hours (Lévi et al. 2010). In contrast, chronotherapy consists in the administration of each drug according to a delivery pattern with precise circadian times in order to achieve best tolerability and best efficacy (Lévi and Okyar 2011). This has mostly involved chronomodulated delivery schedules. Dedicated multichannel programmable pumps have enabled the ambulatory intravenous or intra-arterial administration of multiple drugs according to precisely timed semi-sinusoidal infusion rates, so as to deliver chronotherapy with minimal interference with the daily life of the patient. Oral chemotherapy is also amenable to chronotherapeutic optimization, as suggested in clinical chronopharmacology studies for busulfan, 6-mercaptopurin, and oral fluoropyrimidines (Vassal et al. 1993; Rivard et al. 1993; Etienne-Grimaldi et al. 2008; Qvortrup et al. 2010). A future for oral cancer chronotherapy could stem from chronoprogramed release formulations, since these drug delivery systems allow both chronomodulated drug exposure and nighttime drug uptake without requiring awakening during sleep whenever drug intake should be recommended at night (Spies et al. 2011).

4 Cross Talks Between Chronotolerance and Chronoefficacy

4.1 Experimental Studies

A striking coincidence characterizes the circadian time of best tolerability and that of best efficacy for most chemotherapy drugs in rodents (Fig. 2). Such observation

Table 1 Clock genes' features implicated in cancer survival

Cancer type	Number of subjects	Clock gene	Gene alteration	Clinical outcome	Reference
Colorectal cancer	411	*Clock*	Polymorphism in SNP rs3749474	↑ Survival HR: 0.55; CI 95 %(0.37–0.81); $p = 0.003$	Zhou et al. (2011)
			Polymorphism in SNP rs1801260	↑Survival HR: 0.31; CI 95 %(0.11–0.88); $p = 0.03$	
	19	*CSNKIE*	↓mRNA	↓ Survival ($p = 0.024$)	Mazzoccoli et al. (2011)
		Per1	↓mRNA	↓ Survival ($p = 0.010$)	
		Per3	↓mRNA	↓ Survival ($p = 0.010$)	
	198	*Per2*	↑ PER2 protein	↑ Survival HR: 0.58; CI 95 % (0.40–0.85) $p = 0.005$	Iacobelli et al. (2008)
	202	*Per2*	↓mRNA	Better outcome	Oshima et al. (2011)
Chronic lymphocytic leukemia	116	*Per2 and Cry1*	↓ Per2 mRNA + ↑ Cry1 mRNA	↓Treatment-free survival HR: 3.23; CI 95 % (1.13–9.18); $p = 0.028$	Eisele et al. (2009)
Epithelial ovarian cancer	83	*Cry1 and Bmal1*	↓Cry1 mRNA + ↓Bmal1 mRNA	↓ Survival HR: 5.34; CI 95 %(1.10–25.85); $p = 0.037$	Tokunaga et al. (2008)
Breast Cancer	348	*NPAS2*	↑ mRNA	↑ Survival HR: 0.38; CI 95 %(0.17–0.86); $p = 0.017$	Yi et al. (2010)

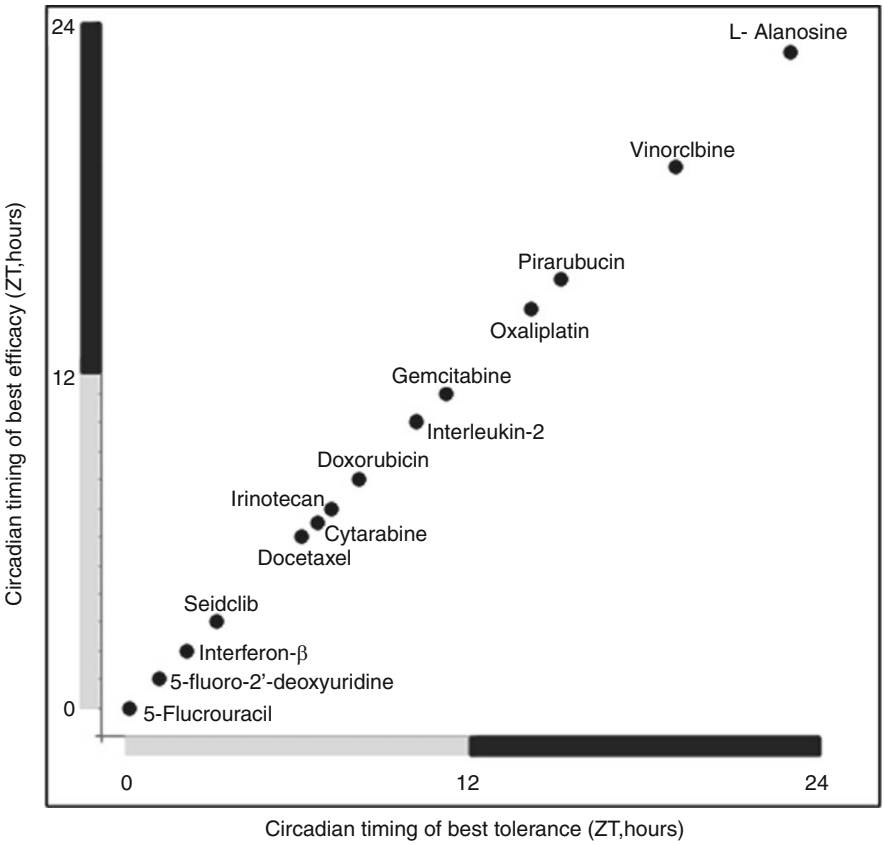

Fig. 2 Coincidence between the circadian time of best tolerance and that of best efficacy for 14 anticancer drugs in rodents

also applies to combination chemotherapy involving two or more anticancer drugs: indeed the best efficacy of the combination treatment is achieved when each drug is administered at its own circadian time of best tolerability, as shown for docetaxel–doxorubicin, for irinotecan–oxaliplatin, and for gemcitabine–cisplatin in tumor-bearing mice (Fig. 3). These results support tight mechanistic links between chronotolerance and chronoefficacy. Moreover, the poor tolerability of the current schedules of these combination chemotherapies and their extensive use in cancer patients further challenge the clinical applications of these experimental chronotherapeutic findings (reviewed in Lévi et al. 2010).

4.2 Clinical Studies

Few clinical studies have investigated the circadian timing concept for chemotherapy administration. A first trial showed that chemotherapy timing was an important

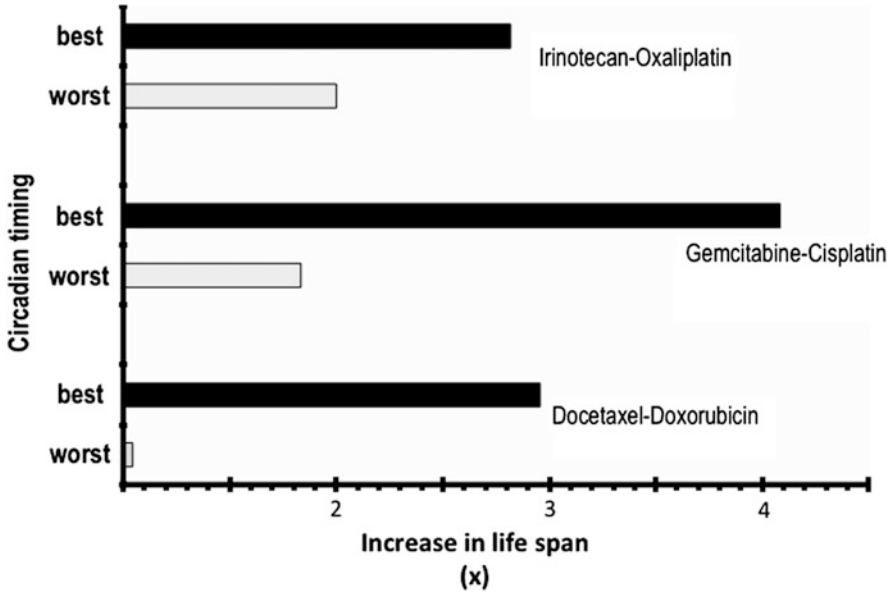

Fig. 3 Relations between chronotolerance and chronoefficacy of three widely used drug combinations in tumor-bearing mice Increase in life span of male B6D2F1 mice with Glasgow osteosarcoma receiving irinotecan–oxaliplatin or gemcitabine–cisplatin and male C3H/He mice with MA13C mammary adenocarcinoma receiving docetaxel–doxorubicin. The figure illustrates the relevance of dosing time of each drug in the combination. Life span was increased several folds when each agent was delivered at the circadian time achieving best tolerability ("best") as compared to that associated with worst tolerability ("worst")

determinant of success in patients with non-small cell lung cancer (Focan et al. 1995). Two trials, each one involving less than 40 patients with advanced ovarian cancers, showed a better tolerability of morning doxorubicin or theprubicin, two DNA-intercalating agents, and late afternoon cisplatin, an alkylating-like drug, as compared to treatment administration 12 h apart (Hrushesky 1985; Lévi et al. 1990). However, the practical difficulties in specifying times of drug administration limited further developments of such approach until the advent of programmable in time drug delivery systems. This dedicated technology enabled intravenous chronomodulated delivery of up to four anticancer drugs without hospitalization of the patient.

Oxaliplatin is the first anticancer drug that has undergone chronotherapeutic development long before its approval for the treatment of colorectal cancer. Indeed this drug was considered as too toxic to pursue its development by the pharmaceutical industry following conventional Phase I clinical testing. Experimental chronotherapeutics studies revealed threefold changes in tolerability according to dosing time in mice (Boughattas et al. 1989). The translation of these findings led to a randomized Phase I study involving 23 patients, 12 of whom received chronomodulated infusion, with peak flow rate at 1600 hours, as compared to 11

treated with constant-rate infusion. Chronotherapy displayed the best safety profile with regard to peripheral sensory neuropathy, the major adverse event of this drug (Caussanel et al. 1990). Interestingly most antitumor activities were recorded among the patients on chronotherapy, a finding subsequently confirmed in patients with metastatic colorectal cancer (Lévi et al. 1993).

The chronomodulated oxaliplatin infusion was then combined with the chronomodulated infusion of 5-fluorouracil–leucovorin (5-FU–LV) with a peak flow rate at 4:00 at night. Five-FU–LV was the reference combination treatment of colorectal cancer. Thus, the first clinical trial that demonstrated the major efficacy of oxaliplatin–5-FU–LV against colorectal cancer involved the chronomodulated delivery of these three agents, the so-called chronoFLO regimen (Lévi et al. 1992). International clinical trials then showed that chronoFLO decreased the incidence of mucosal toxicities fivefold and halved that of peripheral sensory neuropathy as compared to the constant-rate infusion of the same three drugs or their chronomodulated administrations with peak times differing by 9 or 12 h from the initial schedule (Fig. 4) (Lévi et al. 1994, 1997, 2007b). Moreover, in each of these clinical trials, the best tolerated chronotherapy schedule also achieved best tumor shrinkage, based on objective response rate (Innominato et al. 2010). A subsequent international clinical trial involving 564 patients with metastatic colorectal cancer compared 4-day chronoFLO with another 2-day conventional delivery schedule of the same drugs (FOLFOX2 regimen) (de Gramont et al. 1997). Overall survival was similar in both treatment groups. However, chronoFLO significantly reduced the relative risk of an earlier death by 25 % in male patients as compared to FOLFOX2, while the opposite was found in women (Giacchetti et al. 2006). Median survival times differed by 6 months between men and women on chronoFLO, while no gender-related difference was found for the patients on FOLFOX. This strongly supported that the optimal timing of chronoFLO differed between male and female patients. The preclinical studies that were performed in male mice adequately predicted for the optimal timing of the drugs in male patients. In contrast no valid prediction was inferred from male mice to female patients! This clinical finding stressed the need for thorough investigations of sex-related differences in chronotherapeutics. Recent studies along these lines have shown major sex and genetic differences in the chronotolerance of mice for irinotecan, a topoisomerase I inhibitor active against colorectal cancer (Ahowesso et al. 2010; Okyar et al. 2011).

Regional infusions of chronotherapy can also take advantage of the differential circadian organizations of healthy *versus* cancer tissues in a given organ. Such approach is warranted for the medical treatment of liver metastases from colorectal cancer, since this organ is the main one where colorectal cancer cells metastasize. Hepatic arterial infusion (HAI) is performed following the insertion of a catheter into the hepatic artery in order to selectively deliver drugs into the liver and achieve local high drug concentrations (Bouchahda et al. 2011). Our group was first to concurrently administer irinotecan, 5-FU, and oxaliplatin, the three most active drugs against colorectal cancer, into the hepatic artery of patients with liver metastases from colorectal cancer after the failure of most conventional treatment

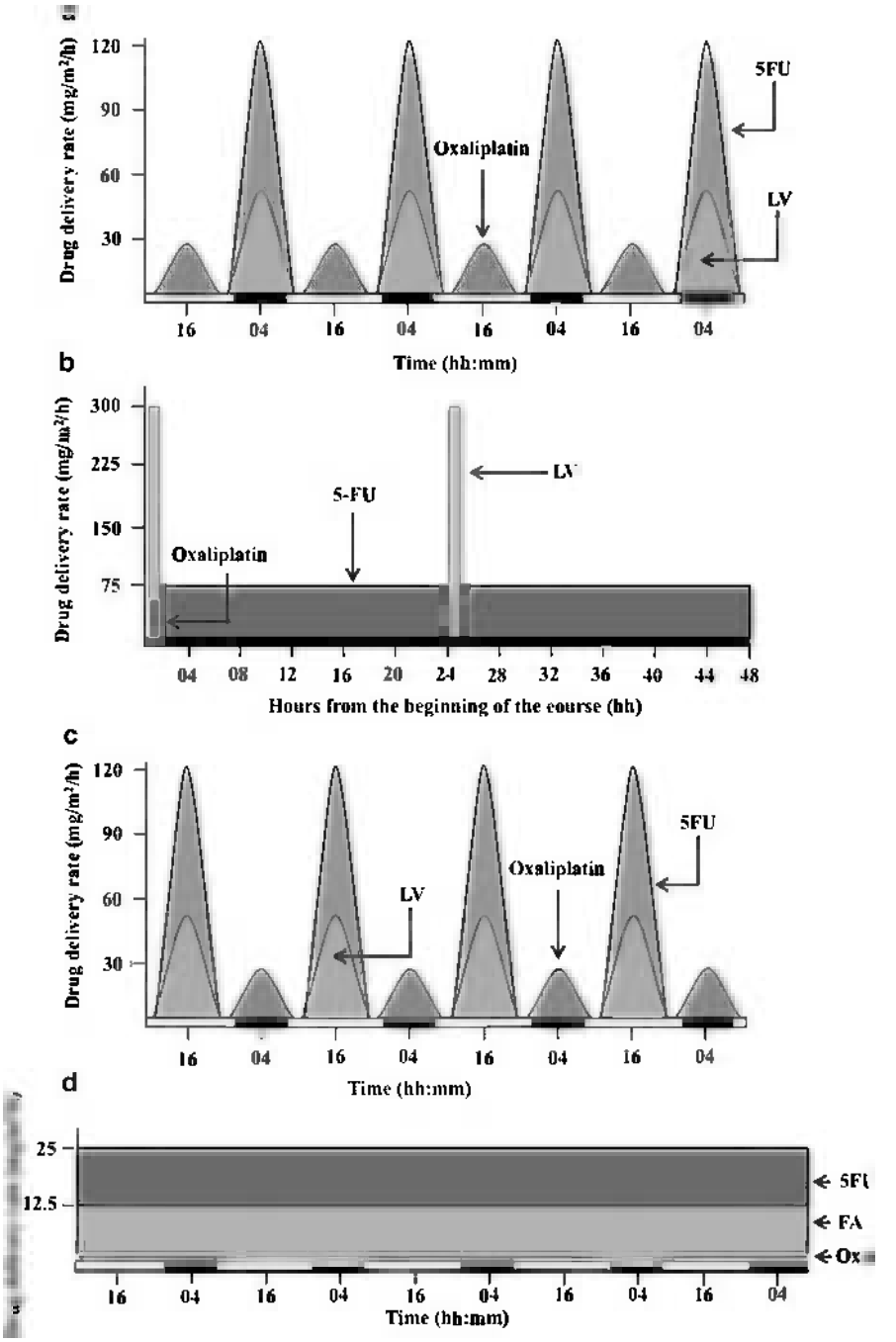

Fig. 4 Combination treatment schedules of metastatic colorectal cancer with 5-fluouroracil (5-FU), leucovorin (LV), and oxaliplatin (**a**) ChronoFLO4. Chronomodulated combination of 5-FU, LV, and oxaliplatin over 4 days, with peak delivery rates programmed at expected times

options. The HAI sinusoidal chronotherapy schedule that we designed proved as safe and effective, with 32 % of the patients displaying an objective tumor shrinkage (Bouchahda et al. 2009). The first European clinical trial of this three-drug HAI regimen has just confirmed the relevance of this approach (OPTILIV, Eudract number 2007-004632-24).

Clinical trials further show that morning radiation therapy tended to cause fewer severe oral mucositis as compared to afternoon radiotherapy in patients with head and neck cancer (Bjarnason et al. 2009). Moreover, the timing of a single high-dose boost of radiations might also be critical for the eradication of brain tumors, as shown in a retrospective study in 58 patients. Thus, morning gamma knife radiosurgery both improved by ~50 % the rate of local tumor control and nearly doubled median survival as compared to afternoon gamma knife radiosurgery (Rahn et al. 2011).

Recent chronochemotherapy findings also challenge the current principle of conventional chemotherapy, where toxicity is considered as a good surrogate endpoint of antitumor efficacy. In other words, the more the toxicity, the better the efficacy! We confirmed this principle in 279 patients with metastatic colorectal cancer receiving conventional chemotherapy with 5-FU, leucovorin, and oxaliplatin (the so-called FOLFOX protocol). Overall survival was significantly predicted by severe neutropenia on FOLFOX. In contrast, severe neutropenia predicted for poor outcome in the 277 patients receiving chronotherapy with the same three drugs (Innominato et al. 2011) (Table 2). Taken together, the clinical chronotherapy data show the relevance of circadian timing of cancer treatments. They confirm the critical role of chronotolerance for chronoefficacy. They further pinpoint the need for tailoring chronomodulated drug delivery schedules according to sex, circadian physiology, and genetic background (Fig. 5).

5 Toward Personalized Cancer Chronotherapy

Chronotolerance and chronoefficacy have been thoroughly investigated in selected mouse strains, in order to minimize intersubject variability. Although two unrelated humans share about 99.99 % of their DNA sequences, the remaining 0.1 % varies and accounts for a large part of intersubject differences in disease risk and drug

Fig. 4 (continued) of least toxicity and best efficacy. The initial version of this reference schedule was administered over 5 days (chronoFLO5) (**b**) FOLFOX2. Conventional combination of these drugs administered without taking circadian timing into account. The only time specifications consist in the sequential timing of oxaliplatin, LV, and 5-FU infusion over the 2 days of the treatment course, while effective start of treatment course depends upon hospital routine organization (**c**) Shifted ChronoFLO4. The peaks in drug delivery rate of each drug are shifted by 12 h with respect to the reference schedule (ChronoFLO4 in panel **a**) (**d**) Constant-rate equidose infusional schedule of 5-FU–LV and oxaliplatin over 5 days. This schedule served as control in a randomized comparison with chronoFLO5

Table 2 Relationship between the incidence of neutropenia (CTC-AE v3) and its prognostic value

Neutropenia		FOLFOX2	ChronoFLO4
None (G0)	% of patients	39.4	67.4
	Median Overall Survival (months)	12.5	19.4
Severe (G3-4)	% of patients	24.7	6.5
	Median Overall Survival (months)	20.7	13.7

Conventional chemotherapy considers that the worse the toxicity experienced by the patient, the better the overall survival. However, this concept seems wrong for chronotherapy, where better survival rates are found among patients who do not experience toxicity. Shaded areas indicate the best survival for each protocol

response. This especially applies to the responses of host and cancer to a given treatment regimen.

The characteristics of the human circadian timing system can also differ according to the individual person. Thus, the timing of several circadian rhythms varied among individuals (Kerkhof and Van Dongen 1996). These changes were commonly related to gender, age, or chronotype (Roenneberg et al. 2007a). Chronotype is defined as the preference to develop our daily routines during the first half of the day (morning types or "larks") or during the second half of the day (evening types or "owls") (Vink et al. 2001). The Munich Chronotype Questionnaire has been used to assess the chronotype in ~55,000 people worldwide (Roenneberg et al. 2007a; for a review see Roenneberg et al. 2013). This epidemiologic study has revealed chronotype differences according to age, gender, and geographical locations, but not ethnicity (Adan and Natale 2002; Roenneberg et al. 2004, 2007a, b; Paine et al. 2006). The "larks" usually display phase-advanced circadian rhythms in rest–activity, body temperature, and melatonin and cortisol secretions as compared to the "owls" (Duffy et al. 1999; Kerkhof and Van Dongen 1996). These interindividual differences in circadian physiology phase seem to translate at the molecular clock level (Cermakian and Boivin 2003). In addition, the endogenous free running circadian period was reported to be shorter in females as compared to males (Duffy et al. 2011).

Indeed striking gender-related differences were found with regard to both tolerability and efficacy of a fixed chronotherapy delivery schedule of oxaliplatin–5-fluorouracil–leucovorin, which proved adequate in men but not in women with metastatic colorectal cancer (Giacchetti et al. 2006, 2012; Lévi et al. 2007b). Moreover, the rhythmic expression of nearly 2,000 genes in the oral mucosa differed between healthy male and female human subjects, with a different timing for many clock-controlled genes relevant for drug metabolism and cellular proliferation (Bjarnason et al. 2001). Besides, several drug metabolism pathways

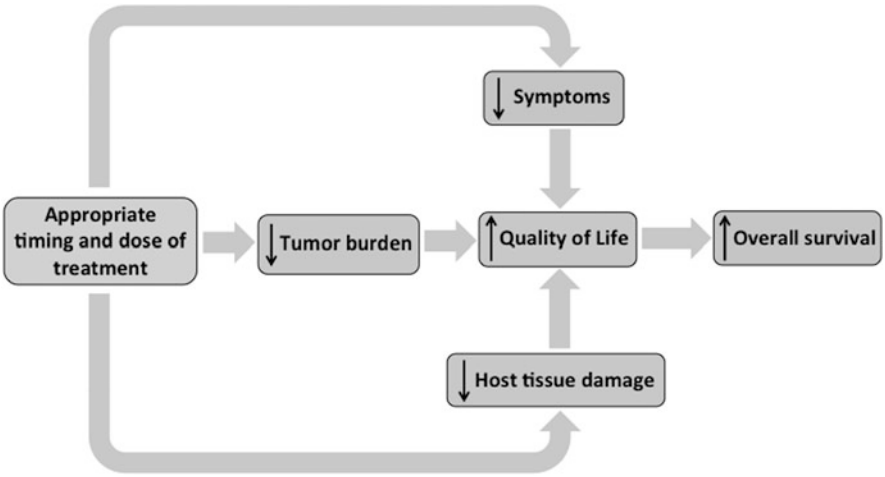

Fig. 5 Theoretical advantages of circadian-based chronotherapy for tolerability, quality of life, and survival The administration of anticancer agents at their adequate timing and safe dose contributes on the one hand to the shrinkage of the tumor burden and on the other hand to a decrease in the side effects of treatment. As a result, patients experience fewer symptoms and display less healthy tissue damage. Altogether, the quality of life of these patients is improved, and this can translate into a favorable impact on overall survival

display strong gender differences (Wang and Huang 2007). Thus, taking full advantage of cancer chronotherapy requires systematic data regarding cancer chronotherapeutics at a molecular level and relevant information regarding the circadian timing system of the individual cancer patient. A systems biology approach to cancer chronotherapeutics currently aims at the development of personalized cancer chronotherapeutics, through the integration of mathematical modeling within research regarding both *in vitro* and *in vivo* chronopharmacology and circadian biomarkers.

5.1 Mathematical Modeling of Chronotherapy Schedules

A combined experimental and mathematical approach has been undertaken in order to propose chronotherapy delivery schedules adapted to the patient genetic and circadian profile (for a review on mathematical modeling of circadian clocks, see Bordyugov et al. 2013). A first step involves the design of a mathematical model of chronotherapeutics and its calibration to experimental data. Once the qualitative and quantitative accuracy of the mathematical models is established, optimization procedures are applied in order to define theoretically optimal chronotherapy schedules, which need to be experimentally validated (Fig. 6).

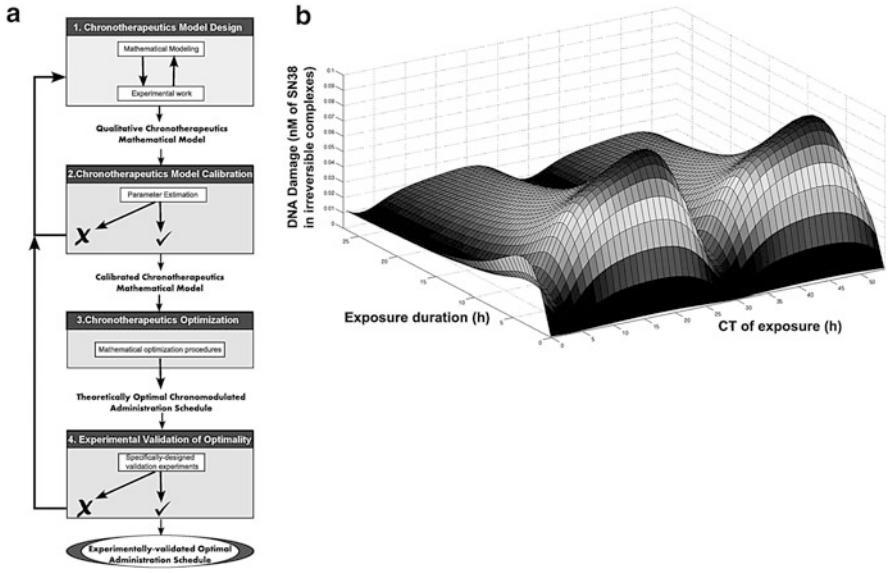

Fig. 6 A combined biological and mathematical approach for chronotherapeutics optimization. (**a**) Chronotherapeutics mathematical models are designed to qualitatively reproduce biological facts as a first step. Then model parameters are estimated by quantitatively confronting the model to experimental data. The calibrated mathematical model is then used in optimization procedures which aim at designing theoretically optimal chronomodulated administration schemes. The final step is the experimental validation of these schemes. (**b**) Simulated toxicity of irinotecan in synchronized Caco-2 cells as a function of drug exposure duration and Circadian Timing of exposure onset (CT). In this mathematical model, toxicity is evaluated by DNA damage on healthy cells. Theoretical exposure schemes consist of the exposure to a fixed cumulative dose of irinotecan, starting at the indicated CT, during the indicated exposure duration, at an initial concentration equal to the cumulative dose divided by the exposure duration. Here, the cumulative dose was set at 500 μM/h

5.1.1 Model Design and Calibration of Circadian Control of Cell Proliferation

In the perspective of modeling cancer chronotherapeutics, the first step is to design models of cell proliferation in the absence of anticancer drugs. Those models include the drug targets, which mainly consist in cell death and cell cycle phase transitions and involve the circadian control of these processes. Models can relate to different scales ranging from single-cell level, where molecular details are in focus, to tissue level, where the model describes the behavior of a cell population.

Molecular models of the cellular circadian clock have been developed since 1965 and have described the interactions between clock genes, which are interconnected in regulatory loops (Goodwin 1965; Merrow et al. 2003). More recent works have focused on the mammalian molecular circadian clock and have taken into account the interplay between clock gene transcription, regulatory effects

of clock proteins, and posttranslational regulations (Leloup and Goldbeter 2003; Leloup et al. 1999; Forger et al. 2003; Relógio et al. 2011). These models have allowed a better understanding of the clock molecular mechanisms especially through clock gene knockout modeling.

Molecular interactions between the circadian clock and the cell cycle through the circadian control of Wee1 and p21 have been mathematically studied using ordinary differential equations (ODEs)-based models (de Maria et al. 2009; Calzone and Soliman 2006; Gérard and Goldbeter 2009). The influence of circadian clock genes knockouts, such as those of *Period*, *Cryptochrome*, and *Bmal1*, on the cell cycle has been studied to further validate the models (de Maria et al. 2009). The converse influence, from cell cycle determinants toward the circadian clock, is still under study. This hypothesis, starting from the remark that transcription is uniformly inhibited during mitosis, has been mathematically explored in a recently published model (Kang et al. 2008).

Several approaches have been undertaken to model cell proliferation and its circadian control at a population scale (Billy et al. 2012). Firstly, physiologically based Partial Differential Equations (PDEs) have been designed in order to describe the fate of cell populations in each phase of the cell cycle (quiescent G0, or proliferating G1, S, G2, M) taking into account the circadian control of death rates and cell cycle phase transitions. Those models enable a theoretical study of cell proliferation. Then, starting from these PDE-based models and with additional assumptions, delay differential equations can also be derived to model circadian-controlled cell proliferation (Foley and Mackey 2009; Bernard et al. 2010). An alternative approach involves agent-based models in which the fate of each cell is computed separately by assuming rules that govern cellular behavior (Altinok et al. 2007; Lévi et al. 2008). Such rules may also include stochastic effects to account for the variability between cells. Those models usually assume high computational cost and do not allow theoretical mathematical analyses.

5.1.2 Mode Design and Calibration: Adding Molecular Chronopharmacokinetics–Chronopharmacodynamics of Anticancer Drugs

A simple statement indicates that pharmacokinetics (PK) is the study of what the body does to the drug (e.g., metabolism, transport), whereas pharmacodynamics (PD) is the study of what the drug does to the body (drug toxicity/efficacy). Circadian rhythms in anticancer drug PK/PD infer from circadian variations of the expression of involved genes. Therefore, in the perspective of optimizing anticancer drug circadian delivery, mechanistic models at the molecular level are required.

Chrono-PK–PD models at the level of a single cell or cell population have been designed for three anticancer drugs in clinical use for colorectal cancer treatment: 5-fluorouracil (Lévi et al. 2010), oxaliplatin (Basdevant et al. 2005; Clairambault 2007), and irinotecan (Ballesta et al. 2011). Those ODE-based molecular models compute the fate of the drug in the intracellular compartment and involve kinetics

parameters that have to be fitted to experimental data measured in the studied biological system (Ballesta et al. 2011). The calibrated models can then be used to optimize the chronomodulated exposure of a cell population to a given anticancer drug. They also allow the integration of relevant polymorphisms in clock and/or drug metabolism genes through the modification of corresponding parameter value.

The subsequent step is the whole-body modeling in order to optimize chronomodulated drug administration and not only exposure. The whole-body models take into account the drug interaction with the entire organism (Tozer and Rowland 2006). They aim at modeling the drug fate from its infusion in the general circulation, to its possible hepatic detoxification, until its delivery to peripheral cells and their response to drug exposure. Hence, such models are generally composed of a blood compartment, a liver one, compartments for the main toxicity targets of the studied drug, and a tumor compartment when relevant. In the perspective of chronotherapeutics optimization, each compartment may contain a model of the intracellular drug chrono-PK–PD. Models of whole-body PK–PD have been proposed for irinotecan (Ballesta et al. 2011) and 5-fluorouracil (Tsukamoto et al. 2001).

The design and parameterization of such models can be done using data in preclinical models such as mouse or rats in which tissue drug concentrations are measured. Indeed, blood concentrations may not vary much with the administration circadian time, whereas tissue concentrations can be highly modified and can play a critical part in the drug chrono-PK–chrono-PD (Ahowesso et al. 2010). A rescaling for cancer patients is needed in which the structure of the model is kept but the parameter values are adapted. The clinical perspective includes the determination of a set of parameters for each patient or class of patients. Then the use of optimization algorithms on this specifically calibrated model allows the design of patient-tailored chronomodulated administration scheme.

In addition to help optimizing drug administration, those models also enable the study of circadian rhythms of proteins that are involved into chrono-PK–PD. This can be relevant in the search of molecular biomarkers that would discriminate between several chronotoxicity classes of mice or of patients (Lévi et al. 2010).

5.1.3 Chronotherapeutics Optimization

In order to efficiently optimize treatment, one should take into account both toxicity, which is here defined as the drug activity on healthy cells, and efficacy, which stands for the drug activity on cancer cells. Therefore, the model should consider at least two compartments, respectively, corresponding to healthy and cancer cells. We described each compartment with the same mathematical model, but with a different parameter set for its calibration. This actually mimics biology, as cancer cells derive from healthy cells and display genetic mutations and epigenetic alterations that speed up or slow down specific molecular pathways. These alterations are thus modeled by an increase or decrease in the corresponding parameter values. For chronotherapeutics optimization, a possible difference

between normal and cancer cells is the disruption of circadian rhythms in the tumor tissue (Ballesta et al. 2011).

Then the therapeutic strategy to be undertaken should be decided. A realistic and clinically relevant strategy consists in maximizing efficacy on cancer cells under the constraint of a maximal allowed toxicity on healthy tissues. The several possible therapeutics strategies can be implemented according to mathematical optimization procedures (Basdevant et al. 2005; Clairambault 2007; Ballesta et al. 2011).

5.2 Circadian Timing System Assessment in Cancer Patients

Minimally or noninvasive procedures represent a critical specification for the determination of the circadian timing system in cancer patients. Nonetheless, the techniques and methods must be safe, reliable, and provide high-quality and informative data about the patient's clocks and their coordination. Whenever circadian physiology is concerned, frequent sampling over several days has been advocated and used in order to provide an insight into the circadian timing system of a patient. These methods include the following:

5.2.1 Rest–Activity Monitoring Through Actimetry

Actimetry was first proposed as the method of choice for reliably, comfortably, and continuously recording the rest–activity rhythm of cancer patients, through a wristwatch accelerometer (Mormont et al. 2000). An adequate definition of its rhythmic characteristics requires two or three 24-h span (Mormont et al. 2000; Ancoli-Israel et al. 2003; Berger et al. 2007), yet our group is currently emphasizing the need for a 1 week monitoring span in order to provide a more reliable assessment of the circadian period and its related parameters. The rest–activity pattern can differ largely among cancer patients with metastatic colorectal, breast, or lung cancer (Mormont et al. 2000; Grutsch et al. 2011; Ancoli-Israel et al. 2001; Innominato et al. 2009). Clinically relevant interpatient differences are best recapitulated in the dichotomy index $I < O$, a relative measure of the activity in bed versus out of bed (Mormont et al. 2000). Indeed $I < O$ identified circadian disruption and was an independent robust predictor of long-term survival outcome in three cohorts of 436 patients with metastatic colorectal cancer (Mormont et al. 2000; Innominato et al. 2009; Levi 2012). Moreover, $I < O$ also identified circadian disruption in patients receiving chemotherapy, and it was also an independent prognostic factor of survival in such condition (Lévi et al. 2010; Innominato et al. 2012).

5.2.2 Body Temperature Monitoring

Body temperature is both a biomarker of the circadian timing system whose pattern is generated by the suprachiasmatic nuclei and an effector of the circadian coordination of peripheral clocks, through the involvement of heat shock and cold-induced proteins (Buhr et al. 2010; see also Buhr and Takahashi 2013). In mice, the circadian amplification of the core body temperature rhythm through meal timing was associated with halving experimental cancer growth (Li et al. 2010). The peak time in core body temperature can further serve as an internal circadian reference for the delivery of chronomodulated cancer therapy (Lévi et al. 2010). Finally, the circadian rhythm in core body temperature can be maintained or disrupted according to both dose and circadian timing of anticancer drugs in mice (Li et al. 2002; Ahowesso et al. 2011).

The core body temperature rhythm has been first determined using a rectal probe eventually connected to an external recorder (Waterhouse et al. 2005; Kräuchi 2002). High values usually occur in the late afternoon while the nadir is reached at late night (Waterhouse et al. 2005). However, this system is neither safe nor convenient for assessing the rhythms in ambulatory cancer patients. In contrast, skin surface temperature can be assessed noninvasively using a radial temperature sensor or skin surface temperature patches (Sarabia et al. 2008; Ortiz-Tudela et al. 2010; Scully et al. 2011). Skin surface temperature patterns are usually opposite to that in core body temperature: the highest point occurs at early night and the lowest point in the early morning, near awakening (Sarabia et al. 2008). Circadian patterns in skin surface temperature, as measured with a thermosensor localized above the radial artery, were determined, together with rest–activity and position patterns, over 7 days in fully ambulatory healthy subjects. The combination of these three biomarkers enabled the computation of an integrated variable called TAP for Temperature–Activity–Position. TAP displayed enhanced stability as compared to each of the three parameters taken separately, thus could best estimate the circadian timing system in real-life conditions and in cancer patients (Ortiz-Tudela et al. 2010). The use of multiple dermal patches on the upper thorax together with rest–activity monitoring also provides relevant information regarding circadian robustness and timing both at baseline and during chemotherapy delivery (Scully et al. 2011; Costa et al. 2013). Finally, a new technology development aims at embedding a telemetry temperature sensor into an implanted vascular access port that is currently used to administer chemotherapy (Beau et al. 2009).

5.2.3 Hormonal Patterns

Cortisol and melatonin rhythms have long been considered as the most robust circadian biomarkers (Veldhuis et al. 1990; Van Someren and Nagtegaal 2007; for a review see Kalsbeek and Fliers 2013). Melatonin secretion usually peaks at early night and it is strongly inhibited by light in humans (Hardeland et al. 2011).

In contrast, cortisol secretion peaks around the waking hours, with lowest values at early night (Clow et al. 2010). Free cortisol can be determined in saliva, so that the 24-h pattern in cortisol secretion can be estimated using salivary samples (Touitou et al. 2009; Mormont et al. 1998). The disruption of the salivary cortisol pattern was found to be an independent prognostic factor for the survival of patients with metastatic breast cancer as well as ovarian and lung cancer (Sephton et al. 2000; Abercrombie et al. 2004). However, no such relation was found for patients with metastatic colorectal cancer (Mormont et al. 2002), indicating a possible cancer specificity of the most relevant circadian biomarkers.

6 Conclusions and Perspectives

Until recently, most efforts in the development of anticancer treatments and strategies have focused on the eradication of cancer cells without paying much attention to the host. The main therapeutic objectives have involved attempts to prevent or impair cell division and/or angiogenesis and/or to induce apoptosis in cancer cells. However, our recent understanding of cancer processes is highlighting a critical role for the tumor microenvironment, thus putting important emphasis on the host cells that infiltrate tumors and surround cancer cells (Hanahan and Weinberg 2011).

Indeed, cancer chronotherapeutics has revealed the major role of circadian timing for both chronotolerance and chronoefficacy. A striking principle is the usual coincidence of chronotolerance and chronoefficacy, which is contrary to the principles that rule conventional cancer treatments. Chronotherapeutics thus allow the design of a new strategy aiming at jointly enhancing host tolerability and antitumor efficacy, through the proper dosing and timing of anticancer medications. Such objective requires thorough consideration to gender, since male and female mice as well as cancer patients can respond differently to the same chronotherapy schedule (Giacchetti et al. 2006, 2012; Lévi et al. 2007b; Ahowesso et al. 2011; Okyar et al. 2011).

Both experimental and clinical data support the relevance of a robust circadian timing system in order to enhance both host control of cancer progression and treatment tolerability. Thus, circadian disruption was shown to accelerate cancer progression in experimental models, and it was an independent prognostic factor of survival in patients with different cancer types and stages (Mormont et al. 2000; Sephton et al. 2000; Innominato et al. 2009). However, treatment itself is able to alter the circadian timing system and this may also convey independent prognostic information regarding the survival of the patient (Ortiz-Tudela et al. 2011; Berger et al. 2010; Savard et al. 2009; Innominato et al. 2012). These data indicate the need to minimize circadian disruption in order to improve chronotherapy efficacy.

Thus, reliable and noninvasive circadian biomarkers, such as those provided with rest–activity and temperature monitoring, are required in the perspective of taking full advantage of the circadian timing system for optimizing cancer treatments.

Biomarkers should provide the quantitative circadian and metabolism data required for adjusting theoretical drug delivery schedules to the individual patient. Large progress has been made in the development of mathematical modeling approaches and their applications to cancer chronotherapeutics. Thus, theoretical models integrate the circadian control of drug metabolism and transport, DNA damage, DNA repair, cell cycle and apoptosis, as well as drug effects on them, based on tight interactions between *in vitro*, *in silico*, and *in vivo* studies, according to systems biology methodology. Recent chronotherapy delivery models can further address issues related to combination chronochemotherapy and treatment strategies. Chronobiotics such as bright light, melatonin, hydrocortisone, meal timing, sleep hygiene, and physical and social activity could further strengthen and/or re-synchronize the circadian timing system (Ancoli-Israel et al. 2011; Seely et al. 2011).

Safety was emphasized as being the major issue that prevents more productive drug development to fight cancer. This chapter shows that chronotherapeutics is critical for jointly improving the safety and the efficacy of anticancer drugs. Indeed, *in vitro*, *in silico*, and *in vivo* models allow a coordinated chronotherapeutic development. Recent technologies now enable the noninvasive recording of circadian biomarkers and the multidimensional assessment of the circadian timing system in an individual patient, while dedicated drug delivery devices or systems can accommodate model-based personalized chronotherapy schedules.

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Pharmacological Modulators of the Circadian Clock as Potential Therapeutic Drugs: Focus on Genotoxic/Anticancer Therapy

Marina P. Antoch and Roman V. Kondratov

Abstract The circadian clock is an evolutionary conserved intrinsic timekeeping mechanism that controls daily variations in multiple biological processes. One important process that is modulated by the circadian clock is an organism's response to genotoxic stress, such as that induced by anticancer drug and radiation treatments. Numerous observations made in animal models have convincingly demonstrated that drug-induced toxicity displays prominent daily variations; therefore, undesirable side effects could be significantly reduced by administration of drugs at specific times when they are better tolerated. In some cases, these critical times of the day coincide with increased sensitivity of tumor cells allowing for a greater therapeutic index. Despite encouraging results of chronomodulated therapies, our knowledge of molecular mechanisms underlying these observations remains sketchy. Here we review recent progress in deciphering mechanistic links between circadian and stress response pathways with a focus on how these findings could be applied to anticancer clinical practice. We discuss the potential for using high-throughput screens to identify small molecules that can modulate basic parameters of the entire circadian machinery as well as functional activity of its individual components. We also describe the discovery of several small molecules that can pharmacologically modulate clock and that have a potential to be developed into therapeutic drugs. We believe that translational applications of clock-targeting pharmaceuticals are twofold: they may be developed into drugs to treat circadian-related disorders or used in combination with existing therapeutic strategies to improve therapeutic index of a given genotoxic treatment via the intrinsic clock mechanism.

Keywords Cancer treatment • Circadian • DNA damage • Pharmacological modulation • Small molecule screen

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1 Molecular Clocks in Mammals

It is well recognized now that the circadian clock regulates almost every important biological process, including sleep–wake cycle, body temperature, metabolism, as well as acute responses to stress (Antoch and Kondratov 2011; Chen and McKnight 2007; Rutter et al. 2002; Sack et al. 2007). A major function of the circadian system is to ensure temporal synchronization of various physiological, behavioral, and metabolic processes within an organism and between an organism and its environment in order to achieve optimal performance. Disruption of proper synchronization results in development of various pathological conditions, including depression and bipolar disease (McClung 2007), sleep (Ptacek et al. 2007), metabolic (Gimble et al. 2011), and cardiovascular disorders (Paschos and FitzGerald 2010). Several epidemiological studies have demonstrated an increased risk of cardiovascular disease, diabetes, and cancer associated with abnormal working schedules resulting in desynchronization between the internal clock and the environment (shift-work, frequent travels across time zones, etc.) (Salhab and Mokbel 2006; Szosland 2010; Wang et al. 2011). Furthermore, studies of animals deficient in individual components of the circadian machinery have identified numerous gene-specific pathologies, including metabolic defects, cancer, and accelerated aging (Kondratov et al. 2007; Takahashi et al. 2008).

During the past 15 years, following the cloning of the first mammalian circadian gene, *Clock* (Antoch et al. 1997; King et al. 1997), enormous progress has been made in deciphering molecular details of clock operation. These advances are summarized in several excellent reviews addressing various aspects of circadian regulatory circuits in different species (Buhr and Takahashi 2013; O'Neill et al. 2013; Minami et al. 2013). Here we will give a brief outline of major mechanisms involved in generation of circadian rhythmicity at the cellular level in order to introduce key players and justify their potential use as perspective therapeutic targets.

At the molecular level, the circadian clock is comprised of a network of transcriptional and translational feedback loops that drive 24-h-based oscillations in RNA and protein abundance of key clock components (Lowrey et al. 2011). At the core of the major circadian loop are two bHLH-PAS domain transcription factors CLOCK and BMAL1 that form a heterodimer to drive rhythmic expression of several genes harboring E-box elements in their promoter region. The negative arm of this loop includes three *Period* (*Per1*, *Per2*, and *Per3*) and two *Cryptochrome* (*Cry1* and *Cry2*) genes that inhibit CLOCK-/BMAL1-driven transcriptional activation. A second loop involves rhythmic regulation of *Bmal1* gene transcription by two nuclear receptors, REV-ERBα (NR1D1) and RORα, both of which are transcriptional targets of CLOCK/BMAL1 and function, respectively, as a repressor and an activator competing for the same regulatory ROR element in the promoter of the *Bmal1* gene. In addition, the CLOCK/BMAL1 dimer regulates transcription of multiple clock-controlled genes with E-box regulatory elements in their promoter regions. Importantly, some of these CLOCK/BMAL1 targets in turn encode transcription factors (such as DBP, TEF, HLF, E4BP4), which work as

transcriptional activators or repressors through a different binding element (D-box) (Schrem et al. 2004). As a result of this multilevel transcriptional regulation, as much as 10 % of mammalian transcriptome displays rhythmicity at the mRNA level (Panda et al. 2002). In mammals, the molecular clocks are operative in virtually all tissues thereby affecting a wide range of physiological and metabolic processes in a tissue-specific manner (Duguay and Cermakian 2009). Notably, the list of clock-controlled genes includes many key regulators of cell cycle, DNA repair, and genotoxic stress response, and circadian oscillations in their concentration and/or activity would be expected to modulate sensitivity to stress and control cell cycle progression under normal and stress conditions (Kondratov and Antoch 2007).

Both positive and negative regulators of the major circadian loop are subject to various posttranslational modifications (phosphorylation, sumoylation, ubiquitination, acetylation), which are important for functional activity, nuclear/cytoplasmic shuttling, and stability of clock proteins. A number of enzymes have been associated with specific modifications of clock proteins, and many of them are now considered as integral clock components. These chemical modifications generate a delay in CRY-/PER-mediated repression to establish the ~24-h rhythms and provide fine-tuning of the system [reviewed in Kojima et al. (2011)]. The complexity of the entire system is further amplified by the involvement of posttranscriptional and epigenetic regulatory mechanisms [reviewed in Lowrey et al. (2011) and Sahar and Sassone-Corsi (2013)] that together with transcriptional and posttranslational mechanisms are integrated into a multifaceted and tightly regulated timing system that renders robustness and precision under constant conditions and provides the necessary plasticity to effectively respond to environmental changes for better adaptation.

One important process that is modulated by the circadian clock is an organism's response to genotoxic stress, such as that induced by anticancer drug and radiation treatments. Pharmacological drugs, UV light, and ionizing radiation are exogenous DNA-damaging agents, which together with endogenous reactive oxygen species (ROS), collapsed replicative forks, and spontaneous lesions of DNA (i.e., cytosine deamination) represent major causes of DNA damages. Under normal circumstances, mammalian cells and tissues may be exposed to DNA damage caused mainly by endogenous factors and, to a certain extent, by UV light. Under unusual conditions, that is, in the course of cancer treatment, various tissues within an organism are exposed to high doses of genotoxic agents. Both chemotherapy and radiation remain major therapeutic approaches directed towards elimination of tumor cells; unfortunately both approaches are nonspecific and do not spare normal cells and tissues causing debilitating side effects.

Numerous animal model observations have convincingly demonstrated that drug-induced toxicity displays prominent daily variations; therefore, undesirable side effects could be significantly reduced by administration of drugs at specific times when they are better tolerated. In some cases, these critical times of the day coincide with increased sensitivity of tumor cells allowing for a greater therapeutic index [reviewed in Levi et al. (2010) and Ortiz-Tudela et al. (2013)]. The results of several clinical trials have confirmed the advantage of chronomodulated therapy over conventional regimens (Innominato et al. 2010). Unfortunately, despite

encouraging results, chronotherapy has not yet become a general clinical practice, explained in part for the following reasons. The vast majorities of observations are of a descriptive nature and lack mechanistic explanation of the findings. Additionally, one might expect that chronomodulated therapy will require treatments at nonconventional times such as the night hours, which would require introducing significant changes in established working schedules of medical personnel. An alternative approach to overcome the latter problem would be to develop pharmaceuticals that reset the molecular clock in sensitive tissues to achieve higher resistance and therefore to allow for a greater therapeutic index. The rationale behind this approach was supported by studies of mice with genetic disruption of either positive or negative components of the circadian transcriptional feedback loop that displayed opposing responses to toxicity induced by chemotherapeutic drug cyclophosphamide suggesting that in vivo responses to genotoxic stresses can be modulated by the functional status of core clock components (Gorbacheva et al. 2005).

2 Circadian Proteins as Modulators of DNA Damage (Genotoxic Stress) Responses

Following exposure to DNA-damaging agents, the cell has multiple response options. The cell may undergo growth arrest allowing for DNA repair, and if the damage is eliminated, the cell may return to its original normal state. If the cell fails to repair the DNA damage, it can be eliminated through apoptosis; alternatively cell proliferation before elimination of DNA mutations potentially leads to neoplasia and tumor development. Finally, the cell may respond by initiating the program of senescence (irreversible growth arrest). The DNA-damaging response option depends on the type of tissue as well as on many extra- and intracellular factors. Recent data suggest that circadian proteins may be involved in this decision-making process. Below, we discuss new findings highlighting the role of circadian proteins at all steps following DNA damage including cell cycle regulation and checkpoint controls, DNA repair, and senescence emphasizing the importance of circadian proteins as novel therapeutic targets.

2.1 The Circadian Clock in Cell Cycle and Checkpoint Control

Circadian gating of the cell cycle was observed decades ago in unicellular organisms (Edmunds and Funch 1969) and was proposed as a mechanism to prevent DNA replication at times of high exposure to UV light to protect genome from the accumulation of UV-induced mutations. Mechanistic links between the circadian clock and the cell cycle have been extensively investigated, and major findings are

summarized in several recent reviews (Khapre et al. 2010; Borgs et al. 2009). Here we will focus on the experimental evidences of the involvement of clock proteins in regulation of cell cycle progression after DNA damage.

Normal cell cycle progression requires several control checkpoints that serve as a surveillance mechanism of DNA lesions induced by endogenous (stalled replication folk, excessive production of ROS, etc.) and exogenous (DNA-damaging drugs, UV light, ionizing radiation) factors. This mechanism provides cells with time to repair the damage, which is critical for maintaining the genome integrity and promote cell survival. In many tumors this pathway is deregulated allowing for uncontrolled proliferation of tumor cells with multiple DNA lesions and leading to aberrant mitosis and cell death through the mechanism known as mitotic catastrophe (Galluzzi et al. 2007). Mitotic catastrophe has been reported as a prominent response of tumor cells to different anticancer drugs (Mansilla et al. 2006).

In normal cells, genotoxic treatments mainly target rapidly dividing cells (bone marrow, intestinal epithelium, and hair follicles), resulting in common side effects, such as myelosuppression, mucositis, and alopecia. All of these tissues are known to harbor functional clocks, and for some, daily variations in the distribution between different phases of the cell cycle have been described (Geyfman and Andersen 2010; Hoogerwerf 2006; Mendez-Ferrer et al. 2009). Therefore, regulation of cell cycle checkpoints by the circadian clock may contribute to protection of normal tissues.

The sensing of DNA damage that results in cell cycle arrest is mediated by two important checkpoint protein kinases, ataxia telangiectasia mutated (ATM) and ATM-Rad3 related (ATR) [reviewed in Smith et al. (2010)]. ATM is activated in response to DNA double-strand breaks and phosphorylates numerous key regulators of cell cycle progression, including CHK2 kinase. It has been reported that circadian protein PER1 can interact with ATM/CHK2 complex and that this interaction is stimulated by ionizing radiation (Gery et al. 2006). Importantly, cells with siRNA suppression of PER1 are impaired in ATM activation and ATM-dependent phosphorylation of CHK2 following radiation treatment. Accordingly, the down regulation of *Per1* in human tumor cell lines makes them more resistant to anticancer drugs (Gery et al. 2006; Gery and Koeffler 2010).

Another interesting interconnection between the components of the circadian clock and cell cycle regulators involves the *Drosophila* homolog TIMELESS (TIM). Although the role of TIM in mammalian circadian clock is not well defined, its association with core circadian proteins PER and CRY has been reported (Barnes et al. 2003; Field et al. 2000). Notably, human TIM interacts with the cell cycle checkpoint proteins CHK1, ATR, and the ATR small subunit ATRIP, and this interaction is stimulated by treatment with DNA-damaging agents such as hydroxyurea and UV light. Moreover, downregulation of TIM by siRNA results in reduced ATR-dependent phosphorylation of CHK1 both under normal and stress conditions (Unsal-Kacmaz et al. 2005). In complex with its non-circadian partner TIPIN (TIM-interacting protein), TIM is involved in regulation of DNA replication and cell cycle progression (Gotter 2003). Functional analyses also revealed the importance of TIM/TIPIN complex in proper checkpoint control after DNA damage (reviewed in Sancar et al. (2010)).

Together, these data identified several components of the circadian clock that can modulate activity of cell cycle regulators under stress conditions and therefore can be viewed as potential targets for pharmacological manipulations directed towards alleviating cellular defects caused by DNA damage.

2.2 Circadian Control of DNA Repair

The first response of a mammalian cell after DNA damage is detected and proliferation has temporarily been restricted, is to repair lesions. In mammals, there are five major systems of DNA repair: nucleotide excision repair (NER) and base excision repair (BER) are responsible for the repair of the single-strand brakes and specific lesions to base/nucleotide; homologous recombination (HR) and nonhomologous end joining (NHEJ) are responsible for the repair of double-strand brakes; and mismatch repair deals with insertions, deletions, or A–G, T–C mismatches. Recent work has established a clear connection between core circadian proteins CRYs and NER.

CRY proteins belong to the family of cryptochrome/photolyases. Most likely, all member of these family evolved from a common ancestor CPD photolyase, an enzyme, which removes UV light-induced cyclobutane pyrimidine dimers from DNA (Kanai et al. 1997). A common evolutional origin suggests functional interaction between the circadian clock and DNA repair systems. Indeed, plants CPD-photolyases have been shown to be able to interact with and regulate the activity of CLOCK/BMAL1 complex similar to mammalian CRYs; moreover, they are able to compensate *Cry*-deficiency and restore circadian oscillation of gene expression in cultured cells and in the liver (Chaves et al. 2011). Mammalian CRYs, on the contrary, do not possess a photolyase-like activity, and the removal of UV-damaged nucleotides in mammals depends solely on NER.

It has been demonstrated that NER of a UV photoproduct displays daily oscillations in the mouse brain and liver with a maximum and minimum values at ZT6 and ZT18, and ZT10 and ZT22 for the brain and liver, respectively (Kang et al. 2009, 2010). Interestingly, in both tissues the maximum of NER activity coincided with the light phase of the cycle, which may reflect the adaptation to UV in the sunlight. In the brain, NER activity also coincides with daily oscillation in the levels of ROS resulting from the brain metabolic activity (Kondratova et al. 2010). This is not surprising given the fact that although the UV light and ROS produce different types of lesions (two major products of DNA oxidation are 8-oxyguanine and thymine glycol), they both are removed by the NER system.

In addition to UV- or oxidative stress-induced lesions, NER system is also capable of removing intra-strand diadducts caused by treatment with cisplatin compounds (cisplatin-d(GpG) and cisplatin-(GpXpG)). Cisplatin is a chemotherapeutic drug widely used to treat various types of cancers, including sarcomas, some carcinomas (i.e., small cell lung and ovarian cancers), lymphomas, and germ cell tumors (Kelland 2007). The repair of cisplatin-induced DNA damage displays daily oscillations in liver extracts with maximum and minimum activity at ZT10 and

ZT22, respectively (Kang et al. 2010). Interestingly, NER activity appeared to be constant in testis, an organ that does not demonstrate prominent circadian oscillation and it is constitutively high in the livers of *Cry*-deficient mice. The latter result indicates that this repair system is activated by the disruption of clock through the *Cry*-deficiency and suggests that the circadian clock downregulates the activity of nucleotide excision repair at certain times of the day.

Mammalian NER is formed by six core repair factors: XPA, XPC, XPF, XPG, RPA, and TFIIH. It has been demonstrated that circadian oscillations in NER activity correlate with the oscillation in protein expression levels of only one of these factors, xeroderma pigmentosum A (XPA) suggesting that XPA is responsible for daily fluctuations in repair activity. Indeed, supplementation of liver lysates isolated at ZT18, when daily repair activity is at its minimum, with XPA restores the level of NER to that observed in the extracts isolated at ZT6. Direct measurement of *Xpa* transcript and protein levels in the liver has showed that both exhibit prominent daily oscillations. The mRNA for *Xpa* peaks at the time of maximum activity of CLOCK/BMAL1 suggesting direct regulation of *Xpa* gene expression by major circadian transactivation complex. In agreement with this, *Xpa* transcript is constitutively high in tissues of *Cry*-deficient mice and does not display oscillations in testis. Thus, the circadian clock regulates nucleotide excision repair in different tissues, most likely, through CLOCK/BMAL1-dependent control of *Xpa* gene expression (Kang et al. 2010).

Circadian regulation of NER was also detected in the mouse skin. Importantly, development of skin tumors after exposure to carcinogens strongly depends on time of exposure and directly correlates with oscillations in NER activity (Gaddameedhi et al. 2011). Together, these findings indicate a physiological significance for circadian regulation of DNA repair. They also underscore the central role of CLOCK/BMAL1 functional activity in modulating cellular response to DNA damage and predict that in contrast to *Cry*-deficient mice, nucleotide excision repair may be significantly reduced in tissues of *Bmal1* knockout animals due to constant low levels of CLOCK/BMAL1-dependent transcriptional activity.

Since skin is the only mammal tissue that is exposed to light (including DNA-damaging UV light), it raises the question of functional significance of the circadian control of NER in other tissues. One possibility is that this functional link is just a relic of the activity that had been advantageous at certain stage of evolution. Alternatively, NER may be involved in protecting cells from oxidative stress as it utilizes similar mechanisms of repair of oxidative lesions. Regardless of the answer, this newly discovered link between the circadian clock and DNA repair system provides an invaluable tool for therapeutic applications.

It is noteworthy that the above-described interactions of PER and TIM with ATM and ATR, respectively, may also affect ATM- and ATR-mediated homologous recombination, which is another mechanism of DNA repair in mammals. Although direct control of a double-strand brake (DSB) repair by the circadian clock has not been demonstrated yet, and the involvement of checkpoint kinases ATM and ATR in these processes is not fully understood (Smith et al. 2010), the fact of their interaction with core circadian proteins PER and TIM respectively allows suggesting their potential involvement in this process.

Indirect evidence for clock control of DSB repair comes from a recent study directed to identification of proteins that regulate checkpoint function, sensitivity to mitomycin C, and efficiency of homologous recombination. The list of 24 strongest candidates includes CLOCK protein; moreover, in subsequent experiments in cells with laser-induced DNA damage, CLOCK was one out of just three proteins that co-localize with γ-H2AX, a well-known marker of DSB sites (Cotta-Ramusino et al. 2011). The latter discovery radically alters the perception of CLOCK protein as exclusively a circadian transcriptional regulator and suggests its involvement in the control of genotoxic stress response not only through transcriptional regulation of target genes but also through a transcription-independent mechanism. One possibility is that DBS DNA repair, which requires chromatin modifications, utilizes the intrinsic HAT activity of CLOCK protein (Doi et al. 2006). Alternatively, CLOCK may recruit other chromatin-modifying or repair enzymes to the sites of DNA damage. For example, SIRT1, a deacetylase which is a well-known regulator of stress response (Rajendran et al. 2011), specifically interacts with CLOCK/BMAL1 complex (Nakahata et al. 2009; Ramsey et al. 2009) suggesting that CLOCK may be necessary for recruitment of SIRT1 to the sites of DNA lesions.

The transcriptional activity of CLOCK/BMAL1 complex can also be important for DNA repair-associated chromatin modifications. Indeed, expression of *Tip60*, a member of MIST family of histone acetylases that is involved in DSB repair in yeast (Sun et al. 2010), is directly regulated by CLOCK/BMAL1 via circadian E-box elements in its promoter (Miyamoto et al. 2008). Regardless of the exact mechanisms, these new findings define the core circadian protein CLOCK as a regulator of several mechanisms of DNA repair that is induced by various genotoxic agents and warrants additional investigation (Kang and Sancar 2009).

2.3 Circadian Clock and Senescence

There is growing evidence that deficiency in certain circadian proteins leads to initiation of the senescence program. Indeed, *Bmal1*−/− and *Clock*/*Clock* mice develop a phenotype of premature aging, the former naturally in life (Kondratov et al. 2006), whereas the latter after challenge by ionizing radiation (Antoch et al. 2008). In agreement with the development of premature aging, increased amount of senescent cells is detected in vasculature of mice with a mutation in the *Per2* gene (Wang et al. 2008) and in the liver, lung, and spleen of *Bmal1*-deficient mice (Khapre et al. 2011). Most likely, accumulation of senescent cells in circadian mutants is associated with stress-induced rather than with replicative senescence. At this moment it is unclear if an increase in senescence is caused by a deficiency in a specific clock protein(s) or by desynchronization of cellular metabolic processes that is induced by deregulation of clock. Most likely both processes contribute to development of senescence as it is observed in different circadian mutants although the severity of the phenotype varies. Stress-induced senescence has been proposed

as one of the mechanisms for tumor suppression (Campisi 2005), and it is likely that regression in size in many tumors in response to chemotherapy results from activation of their senescence program. In this respect, involvement of some circadian proteins in development of senescent phenotype provides additional argument in support of their therapeutic potential.

It is important to emphasize though that many senescent cells retain their metabolic activity and can secrete many factors affecting an organism's physiology including those that promote tumorigenesis. Such a dual role of senescence in promoting both tumor suppression and tumor development may depend on conditions (normal versus stress-induced) as well as type of tissue and may explain an existing controversy regarding the role of clock proteins in tumorigenesis. Indeed, mice with mutations in circadian protein PER2 were reported to display a cancer-prone phenotype resulting from decrease in *p53*-dependent apoptosis following exposure to ionizing radiation (Fu et al. 2002). At the same time, *Clock* mutant mice respond to ionizing radiation by accelerating their aging and do not develop tumors (Antoch et al. 2008), whereas the deficiency in both CRY proteins rescues tumor-prone phenotype of *p53*-null mice (Ozturk et al. 2009). It is possible that, depending on the type of circadian deficiency and the methods used to induce tumors in experimental mouse models, exposure to genotoxic agents and activation of senescent program in normal cells can stimulate tumor growth and at the same time suppress growth of transformed cells.

In summary, recently established interaction between the components of molecular clock, cell cycle regulation, genotoxic stress response, and tumorigenesis opens novel perspectives both in anticancer treatment and tumor prevention. More studies are needed to refine molecular mechanisms of clock-mediated regulation of stress response pathways and to resolve multiple contradictions currently existing in the field. However, the very fact of the established cross talk among these metabolic processes underscores the importance of circadian proteins as targets for therapeutic applications.

3 Search for Pharmacological Modulators of Circadian Clock by High-Throughput Chemical Screen

High-throughput screening of libraries of small organic molecules is one of the most effective tools for the discovery of bioactive compounds. The pioneer work of Balsalobre et al. (1998), which demonstrated that cultured cells could display rhythms in circadian gene expression after short treatment by high serum concentration, initiated a cascade of experiments performed in different laboratories that resulted in the identification of several compounds that could affect circadian function. Thus, circadian oscillation in cultured cells can be induced by the glucocorticoid receptor agonist, dexamethasone (Balsalobre et al. 2000a), by the activator of adenylate cyclase, forskolin (Yagita and Okamura 2000), phorbol-12-myristate-13-acetate

(PMA), fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin, calcium ionophore calcimycin (Balsalobre et al. 2000b), endothelin (Yagita et al. 2001), glucose (Hirota et al. 2002), and prostaglandin E2 (Tsuchiya et al. 2005). Furthermore, several intracellular small molecules such as NAD (Nakahata et al. 2009; Ramsey et al. 2009), heme (Raghuram et al. 2007; Yin et al. 2007), and cAMP (O'Neill et al. 2008) can function as circadian modulators. The differences in rhythm-inducing properties, which was revealed by comparative quantitative analysis of ten individual signaling compounds (Izumo et al. 2006), indicated that all of them likely exert their action through different pathways. Together, these work provided a proof-of-principle for performing large-scale screens in a cell-based assay to identify more specific chemicals that can modulate regulators of the circadian oscillator. Two types of experimental approaches have been recently explored. The first one is based on a real-time recording of a circadian reporter activity in cells with synchronized circadian rhythms (by serum shock, dexamethasone, or forskolin). In this experimental setup, chemical compounds are tested for their effect on basic circadian parameters (circadian period, amplitude, and phase of rhythmicity). The second approach involved search of small molecules that modulate the steady state activity of the core clock proteins in nonsynchronized cells. Both approached that are described below led to identification of novel modulators of the circadian clock that have a potential to be developed into pharmaceutical drugs.

3.1 Small Molecules Affecting Circadian Parameters in Cultured Cells

This screening paradigm was first tested in a small-scale screen of 1,280 structurally diverse chemicals present in commercially available Library of Pharmacologically Active Compounds (LOPAC, Sigma). It resulted in identification of small molecule inhibitors of glycogen synthase kinase 3β (GSK-3β) that mediated a shortening of the period of circadian oscillation in osteosarcoma U2OS cells stably expressing *Bmal1*-Luc reporter (Hirota et al. 2008). In mammals, GSK-3β has been previously identified as a kinase that directly phosphorylates several core clock proteins including PER2, CRY2, REV-ERBα, CLOCK, and BMAL1 (Harada et al. 2005; Iitaka et al. 2005; Sahar et al. 2010; Yin et al. 2006; Spengler et al. 2009), which leads to their degradation (in case of CRY2, CLOCK, and BMAL1), increased nuclear translocation (PER2), or stabilization (REV-ERBα).

Further development of this approach was applied to a circadian screen of ~120,000 uncharacterized compounds and resulted in identification of a small molecule (named longdaysin) that potently lengthened the circadian period in a variety of cultured cells and in explants of mouse suprachiasmatic nucleus (SCN), the site of central pacemaker in mammals (Hirota et al. 2010). Importantly, longdaysin also affected the circadian period in vivo in transgenic zebra fish expressing circadian reporter. The combination of pharmacological, mass

spectrometry, and siRNA-mediated gene suppression approaches revealed that longdaysin targets several protein kinases, CK1δ, CKIα, and ERK2. Whereas CK1δ and ERK have been identified previously as clock-regulating kinases, the role of CK1α in circadian control was unknown. It appeared that CK1α directly phosphorylated PER1 protein and similar to CK1δ promotes its degradation (Hirota et al. 2010).

In addition to several smaller screens that specifically focused on the effects of protein kinase inhibitors (Isojima et al. 2009; Yagita et al. 2009), the above-described large-scale screens mainly identified compounds affecting period of circadian oscillations. A recent novel screen targeted other circadian parameters, such as amplitude of rhythmicity (Chen et al. 2012). This study was performed on immortalized mouse fibroblast cells derived from *Per2*::*lucSV* reporter mice, synchronized by forskolin treatment, and involved testing of ~200,000 synthetic small molecules. Several hits were identified from these screens that in addition to period shortening caused a significant increase in the amplitude of oscillation, which correlated with an increase in expression of two clock output genes, *Dbp* and *Rev-Erbα*. An interesting class of compounds, which have not been previously characterized, mediated an acute induction of *Per2*-driven luciferase signal followed by significant phase delay of oscillation, which was somewhat similar to the effect of forskolin in SCN slices (O'Neill et al. 2008). Further analysis revealed that these chemicals indeed induced intracellular cAMP levels. Their effect on circadian oscillation appeared to be very complex, which likely reflects the fact that cAMP is involved in regulation of numerous pathways.

More recently, circadian phenotypic screen identified a small molecule that specifically acts on core circadian protein CRY preventing its ubiquitin-dependent degradation, which results in lengthening of the circadian period (Hirota et al. 2012). Importantly, in addition to its ability to affect circadian parameters, this compound, named KL001, provides a tool for study the regulation of CRY-dependent physiological processes, such as gluconeogenesis. It has been reported that in the liver, CRY proteins negatively regulate transcription of two genes, phosphoenolpyruvate carboxykinase 1 (*Pck1*) and Glucose-6-phosphatase (*G6pc*), which encode rate-limiting enzymes of gluconeogenesis (Lamia et al. 2011; Zhang et al. 2010). Consistent with this, treatment of mouse primary hepatocytes with KL001 effectively repressed glucagon-mediated induction of *Pck1* and *G6pc* as well as glucose production (Hirota et al. 2012). These data indicate that KL001 may be considered as clock-based therapeutics for treatment of diabetes.

Together, the above-described screens identified a number of compounds belonging to different chemical classes that affected circadian oscillatory parameters including several molecules with unknown biological function. The fact that they display diverse activities and affect different circadian parameters suggests multiple molecular mechanisms involved. These compounds have served as important tools for probing different regulatory mechanisms involved in circadian regulation and have already led to identification of novel players in circadian circuit, such as CK1α (Hirota et al. 2010) with a potential to discover more. They also have a potential to be developed into drugs for treating circadian-related

pathologies. Thus, many of circadian disorders are associated with dampened clock, as for example, ones related to *Clock*Δ*19* mutation (Marcheva et al. 2010; Vitaterna et al. 2006). In this respect, the small molecules identified by these screens and tested for their ability to restore amplitude of oscillation in fibroblasts as well as in pituitary and SCN derived from *Clock*/+ mice represent perspective prototype drugs (Chen et al. 2012). In addition, the kinase regulators affecting circadian function via control of the phosphorylation status of PER may be developed into pharmaceuticals for treatment of pathologies such as familial sleep phase syndrome (FASPS) (Vanselow et al. 2006).

3.2 Screen for Small Molecules Affecting the Functionality of CLOCK/BMAL1 Transcriptional Complex

In addition to their roles as components of a molecular circadian oscillator, many (if not all) core clock proteins have been ascribed clock-independent physiological functions (Yu and Weaver 2011). The impairment of any of these functions in experimental systems leads to development of various pathologies that often are related to various human diseases. Therefore, the identification of functional small molecule regulators of individual clock proteins that may not be necessarily linked to their circadian function may provide a more specific therapeutic drug. Such an approach has been recently used in a screen for modulators of CLOCK/BMAL1 transcriptional activity (Hu et al. 2011). The rationale for this approach was based on a previously published work that linked the acute response of genotoxic treatment in different circadian mutant mice with the functional status of the major circadian regulator, CLOCK/BMAL1 transcriptional complex (Gorbacheva et al. 2005). These studies have demonstrated that the different types of circadian mutants (*Clock* mutant mice, *Bmal1* knockout, and *Cry* double-knockout mice), although all behaviorally arrhythmic, displayed an opposite response to toxicity induced by the chemotherapeutic drug cyclophosphamide (CY). The animals with a deficiency in circadian activators (CLOCK and BMAL1), which results in constant low levels of clock-controlled gene expression, were extremely sensitive to CY-induced toxicity, whereas mice with deficiency in circadian repressors CRYs, which results in constant high levels of CLOCK/BMAL1-mediated transcription, were very resistant to the treatment. These data highlight the importance of identifying the specific components of the circadian mechanism that are being targeted by circadian modifiers in order to elicit the desired therapeutic response and indicate that for many therapeutic applications, it is important to recognize not only the fact of circadian disruption but also to identify deficiency of which component caused this disruption. This data also allowed to define circadian transcriptional activators as potential targets for pharmacological modulation aimed at protecting normal tissues from damage induced by genotoxic treatments.

A small-scale screen for modulators of CLOCK/BMAL1-dependent transactivation was performed in a readout system based on mouse fibrosarcoma L929 cells expressing high levels of endogenous CLOCK and BMAL1 and stably expressing *Per1*-driven luciferase reporter. Two commercially available libraries, LOPAC (Sigma, 1,200 compounds) and Spectrum (library of 2,000 natural compounds, MicroSource Discovery, Inc), were used to screen for activators and inhibitors of CLOCK/BMAL1-mediated expression of *Per1* gene (Antoch and Chernov 2009). Importantly, this screen identified several known regulators of circadian function such as glucocorticoids, 2-methoxyestradiol, forskolin, PKC, and p38 MAPK inhibitors as well as some hits identified by circadian-driven approach (Hirota et al. 2008), all of which validated the feasibility of the approach. It also identified several chemicals that have not been previously linked to circadian function including the organic selenium compound, L-methyl selenocysteine (MSC) (Hu et al. 2011).

Selenium is an essential trace element that has two major clinical applications: tumor prevention and protection against DNA damage induced by anticancer therapy. Studies in cell-based model systems, as well as several clinical trials, have conclusively demonstrated that selenium supplementation ameliorates radiation-induced mucositis in mice treated with fractionated doses of ionizing radiation (Gehrisch and Dorr 2007) as well as radiation-induced diarrhea in treatment of patients with cervical and uterine cancers (Muecke et al. 2010). It has been determined that the observed increase in *Per1*-driven luciferase in cells treated with MSC is caused by selenium-mediated transcriptional upregulation of the *Bmal1* promoter resulting in increase in BMAL1 protein and presumably of the transactivation potential of CLOCK/BMAL1 complex. Mechanistically, the effect of selenium was attributed to its ability to prevent binding of glucose-inducible gene 1 (*Tieg1*), an Sp1 family transcription repressor involved in *Bmal1* regulation (Hirota et al. 2007), to Sp1-binding sites in *Bmal1* promoter (Hu et al. 2011). Importantly, the effect of selenium on BMAL1 protein abundance was detected not only in cells but also in vivo in mice that receive the compound either through a single injection or systemically via gavage or selenium-supplemented diet. Interestingly, the in vivo effect of selenium was found to be tissue-specific in that selenium-induced changes in BMAL1 were detected in the liver, but not in the SCN; consistent with this, no changes in circadian behavioral parameters were detected. This finding is reminiscent of characteristics of small molecules identified in circadian-based screens. Originally identified as circadian modulators in synchronized fibroblasts, they often display tissue-specific variations when tested in explants derived from different tissues (Chen et al. 2012). From the therapeutic standpoint, this may present a huge advantage rather than a weakness as it allows for modulation of response to genotoxic treatments in a tissue-specific manner without disturbing the central clock.

Notably, through the upregulation of BMAL1, selenium administration alleviated CY-induced toxicity in drug-sensitive *Clock* mutant mice as displayed by increase in their survival rate and decreased levels of myelosuppression. In contrast, selenium failed to produce these ameliorating effects in mice with genetic

disruption of the *Bmal1* gene, thus confirming that the rescuing effect of selenium in vivo is mediated, to a large extend, through BMAL1. Together, these findings provide a plausible mechanism behind tissue-protective effects of selenium by linking it to circadian regulation of gene expression and suggest that selenium is capable of tuning circadian transcriptional machinery to the higher activity, which is associated with maximum resistance by upregulating BMAL1 expression.

Although the exact mechanism, by which the increase in CLOCK/BMAL1 activity ameliorates CY-induced toxicity, is not fully understood, this work presents the first example of protection of normal tissue from drug-induced damage through the components of the molecular clock. Based on known targets of CY-induced toxicity, one could predict that an important factor in determining the in vivo drug response and host survival in clinical therapy is CLOCK/BMAL1-dependent modulation of the lymphocyte survival/recovery rate. Consistent with this, studies with *Bmal1*−/− mice revealed the involvement of BMAL1 in differentiation of pre-B to mature B cells, although direct molecular targets are still not known (Sun et al. 2006). Another potential mechanism may involve BMAL1-dependent regulation of ROS homeostasis (Kondratov et al. 2006), which would protect against excessive accumulation of ROS in response to genotoxic stress and thereby ameliorate drug-induced tissue damage. However, regardless of a precise molecular mechanism, the reported ability of selenium to modulate activity of circadian transcriptional complex, without affecting central clock, opens new possibilities for clinical applications. If previously clock-targeting pharmaceuticals were considered mostly as resetting agents (with the goal to reset molecular clocks in drug- and radiation-sensitive tissues to times of higher resistance to genotoxic treatment), selenium compounds demonstrate the ability to minimize the damaging effects of genotoxic treatments by a constant upregulation of circadian transcriptional activators in a tissue-specific manner.

Another example of the therapeutic value of drugs targeting CLOCK/BMAL1 functional activity came from series of studies in *Cry*-deficient mice. It has been found that disruption of the circadian clock by a *Cry* mutation in *p53*-null background makes them more sensitive to UV light-induced apoptosis (Ozturk et al. 2009). Mechanistically, this increase accounted for CLOCK/BMAL1-mediated upregulation of *p73*-dependent apoptosis. It was shown that in the absence of *p53* (the primary tumor suppressor) (Lowe et al. 1994), downregulation of *Cry* enhances expression of another member of the *p53* family, *p73* (Stiewe 2007), and subsequently enhances UV-mediated apoptosis and elimination of damaged cells and reduces risk of cancer. Upregulation of *p73* in the absence of *Cry* correlates with increased levels of *Early growth response 1* (*Egr1*) gene, which works as a positive activator of *p73* (Yu et al. 2007), and which itself is directly regulated by CLOCK/BMAL1 transcriptional complex. Consistent with this, *Egr1* levels are constantly elevated in *Cry*-deficient cells, and this upregulation is reversed by downregulation of BMAL1 (Lee and Sancar 2011). Chromatin immunoprecipitation experiments have also demonstrated that BMAL1 binds the *Egr1* promoter and that although both positive (EGR1) and negative (C-EBPα) regulators of *p73* are present on *p73* promoter, only EGR1 remains bound upon exposure to UV light (Lee and Sancar 2011).

Importantly, when tumor xenografts induced by oncogenically transformed *p53−/−* and *p53−/−Cry−/−* cells were treated with oxaliplatin, a chemotherapeutic drug widely used to treat many forms of metastatic cancers, it suppressed tumor growth in *p53−/−Cry−/−* tumors but display no therapeutic effect on the growth of *p53−/−* tumors. Together, these data provide a plausible mechanism for the sensitization of tumor cells that are often deficient in *p53* function to cytotoxic drugs through activation of *p73*-dependent apoptotic program mediated by CLOCK/BMAL1 circadian regulators (Lee and Sancar 2011).

4 Concluding Remarks

In conclusion, we would like to emphasize that cancer treatment involves frequent use of highly toxic compounds that commonly induce severe adverse effects resulting in reduced efficacy of therapy, creating risks of acquisition of additional diseases and reducing quality of life of cancer patients. In this respect, clock-targeting pharmaceuticals represent a huge potential that is still underestimated and therefore not yet developed. However, to fully exploit circadian mechanism for increasing therapeutic index of anticancer treatment, it is important to define the functional status of clock proteins in tumor cells and tumors. Whereas the functionality of molecular clocks in normal tissues has been extensively studied and recently resulted in significant breakthroughs, our knowledge of circadian status of tumor cells and tumors is still sporadic and often controversial. There is growing evidence that at least in part, the controversy may arise from the fact that circadian proteins play different roles in normal and tumor cells. Thus, both CLOCK and BMAL1 are positive regulators of the cell cycle in normal cells such as hepatocytes (Grechez-Cassiau et al. 2008), hair follicles (Lin et al. 2009), and embryonic fibroblasts (Miller et al. 2007). At the same time, it has been reported that BMAL1 is necessary for *p53*-dependent growth arrest in human tumor cell lines in response to DNA damage. Accordingly, suppression of *Bmal1* decreases induction of *p21*, impairs growth arrest, and sensitizes tumor cells to DNA-damaging agents (Mullenders et al. 2009). Consistent with its role as a mediator of growth arrest, BMAL1 is epigenetically silenced in several hematological malignancies, which may contribute to tumor growth (Taniguchi et al. 2009). These controversial data indicate that more work is required to better understand mechanistic differences in circadian modulation of stress response pathways in normal and tumor cells. However, even the first examples of such differential regulation are encouraging as they suggest the potential to develop therapeutic approaches that target an individual clock component that elicits both an in increased resistance of normal cells and increased sensitivity of tumors.

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Light and the Human Circadian Clock

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Abstract The circadian clock can only reliably fulfil its function if it is stably entrained. Most clocks use the light–dark cycle as environmental signal (zeitgeber) for this active synchronisation. How we think about clock function and entrainment has been strongly influenced by the early concepts of the field's pioneers, and the astonishing finding that circadian rhythms continue a self-sustained oscillation in constant conditions has become central to our understanding of entrainment.

Here, we argue that we have to rethink these initial circadian dogmas to fully understand the circadian programme and how it entrains. Light is also the prominent zeitgeber for the human clock, as has been shown experimentally in the laboratory and in large-scale epidemiological studies in real life, and we hypothesise that social zeitgebers act through light entrainment via behavioural feedback loops (zeitnehmer). We show that human entrainment can be investigated in detail outside of the laboratory, by using the many 'experimental' conditions provided by the real world, such as daylight savings time, the 'forced synchrony' imposed by the introduction of time zones, or the fact that humans increasingly create their own light environment. The conditions of human entrainment have changed drastically over the past 100 years and have led to an increasing discrepancy between biological and social time (social jetlag). The increasing evidence that social jetlag has detrimental consequences for health suggests that shift-work is only an extreme

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form of circadian misalignment, and that the majority of the population in the industrialised world suffers from a similarly ‘forced synchrony’.

Keywords Chronotype • Entrainment • Sleep • Zeitgeber • Zeitnehmer • Free-running period • Clock evolution

1 Introduction

Chronobiology investigates temporal structures, rather than the linear passing of time. The German language discriminates between linear time (*Zeit*) and temporal structures (*Zeitraum*; German for ‘time-space’; plural *Zeiträume*). Life on Earth is influenced by four *Zeiträume*, the tides (12.5 h), the day (24 h), the lunar month (28.5 days) and the year (365.25 days). One or more of these are represented by endogenous clocks in most organisms. Here we focus on the human circadian clock, specifically on the importance of light for the process that actively synchronises its endogenous day to that of the environment (entrainment).

Circadian clocks create an internal representation of the external *Zeitraum* day by generating a dynamic milieu at the cellular and the organismal level that oscillates with a circa-24-h rhythm. Beyond their function of generating daily rhythms, circadian clocks are sensors for environmental information that allows them to remain entrained to the regular changes of day and night, of light and dark, of warm and cold, of humidity and of all the resources that depend on these environmental changes (availability of food, presence of enemies and/or competitors etc.).

These regular changes have provided the selection pressures that have led to the development of circadian clocks very early on in evolution. The genes that are essential to make these endogenous clocks tick are not conserved across kingdoms (prokaryotes, unicellular eukaryotes, fungi, plants and animals), suggesting that these programmes have evolved several times during evolution. The recent discovery of non-transcriptional circadian oscillators (O’Neill and Reddy 2011; O’Neill et al. 2011) suggests, however, that a basic metabolic rhythm-generator may be ancestral to all circadian clocks, and that the specific transcriptional–translational mechanisms represent adaptations in the respective phyla.

1.1 A Clock with Many Names

The circadian system is referred to by many terms: oscillator, clock, pacemaker or temporal programme. The term ‘clock’ was used early on to describe the circadian programme and has strongly influenced our concepts, experimental approaches and interpretations of results (Roenneberg et al. 2008). Yet, the notion of a ‘clock’ evokes distinct associations: its hands always move with the same pace; it reliably represents time that we consult to take appropriate actions at the appropriate times;

its mechanism has to be compensated against temperature changes to keep the correct time. All these qualities of a physical clock were also associated with their biological counterparts.

In many cases, biological clocks are indeed used to 'read' the correct time, for example, in the dance 'language' of bees (Frisch 1967), the orientation of migrating birds (Gwinner 1996; Kramer 1952) or on the annual/seasonal time frame in photoperiodism (Bünning 1960). Temperature compensation is also a quality of biological clocks, since their free-running period does not change significantly with temperature (Hastings and Sweeney 1957); this quality was thus defined as one of the basic circadian properties (Roenneberg and Merrow 1998). Despite these apparent similarities, circadian clocks are not just mirror images of physical clocks. The velocity at which circadian clocks progress through their daily cycles is most probably not constant (Pittendrigh and Daan 1976; Roenneberg et al. 2010b), and temperature compensation of the free-running period does not mean that circadian clocks are insensitive to temperature changes; on the contrary, most of them can perfectly entrain to temperature cycles.

The circa-24-h rhythmicity is generated at the cellular level by molecular oscillators—based on transcriptional–translational mechanisms (Roenneberg and Merrow 2003), on metabolic feedback loops (O'Neill and Reddy 2011; O'Neill et al. 2011), or on their interaction. Molecular oscillators are not necessarily a circadian clock, which constitutes an organism's circadian programme (Pittendrigh 1993). In single-cell organisms, they fulfil the role of such a programme, although even at that level, several molecular oscillators can form a network (Baggs et al. 2009; Roenneberg and Morse 1993; Roenneberg and Merrow 2003). Although molecular oscillators are also found in virtually every cell of higher plants (Thain et al. 2000) and animals (O'Neill and Reddy 2011; Schibler et al. 2003), their circadian programme is an emergent property of the interactions between these oscillators. So, at all levels—from cells to organism—the circadian programme, which coordinates all functions to do the right thing at the right time within the 24-h day, involves many interacting oscillators, which all are part of the active synchronisation process called 'entrainment'.

1.2 Zeitgeber

Any environmental factor that varies across the 24-h day can potentially serve as an entraining signal (zeitgeber; German for 'time giver'). The evolutionary oldest clocks known are those in cyanobacteria (Johnson et al. 1996), photosynthesising prokaryotes. For photosynthesising organisms, light is both energy resource and zeitgeber. Thus, the oldest zeitgeber is an energy source and is some form of 'food'. As more clocks will be discovered in organisms that are not exposed to light–dark (LD) cycles and have so far been thought to be clock-less (e.g. those that live in the gut of a host), we may find that the rhythmic availability of 'food' can act as the primary zeitgeber. The single-cell organism *Lingulodinium* (former *Gonyaulax*) entrains to

changing nutrient concentrations (e.g. nitrate; Roenneberg and Rehman 1996), and the clocks in mammalian liver cells synchronise to food (Stokkan et al. 2001). In contrast, the clock in the mammalian central pacemaker (the suprachiasmatic nucleus; SCN) appears to only use light as zeitgeber (Yamazaki et al. 2000), surrogated by transmitters released from collaterals of the optic nerves (van Esseveldt et al. 2000). Temperature is also a universal zeitgeber for circadian oscillators from single-cell organisms (Edmunds 1984) and fungi (Merrow et al. 1999) to tissue clocks in mammals (Brown et al. 2002; Buhr et al. 2010).

As much as the circadian clock of an organism has to entrain to its environment, the many cellular oscillators within an organism have to synchronise to their rhythmic internal milieu. In plants (Thain et al. 2000) and even in insects (Plautz et al. 1997), many entrain directly to light (i.e. by external time), while in mammals, for example, many signals can act as internal time cues (factors that fluctuate in the bloodstream, neuronal transmitters or body temperature; Dibner et al. 2010). Although any environmental factor that oscillates in a 24-h rhythm can act as a zeitgeber for different oscillators and under different conditions, light is the zeitgeber most abundantly used by circadian clocks. The reason for this dominant role is because light (and darkness) is responsible for all other environmental rhythms, and it is therefore the primary and most reliable source of information about time-of-day. Note that entrainment is an active process of the circadian system; the clock therefore *entrains to* rather than being *entrained by* a zeitgeber.

1.3 Input Feedback Loops: Zeitnehmer

The clock's rhythm generation and its sensory function are inseparable. Circadian programmes modulate their own input pathways at all levels—from the primary and secondary components of the reception pathway down to the molecules of the oscillator mechanism itself (Roenneberg and Merrow 2000, 2003). The environmental signals that allow the clock to actively entrain to the daily structure of the world are rhythmic and so is the machinery that senses them. We have therefore called these feedbacks, which are both input and output of the circadian system, *zeitnehmer* (German for 'time taker'; McWatters et al. 2000; Roenneberg et al. 1998).

The dual role of circadian clocks as rhythm generators and as sensors is especially obvious in the mammalian SCN. It generates circadian rhythms in many of its cells but also as a tightly coupled neuronal network and entrains to the LD cycle via retinal inputs (Rea 1998). As such, the SCN, which is often called the central pacemaker of the mammalian circadian system, serves predominantly as a relay station that transduces the information of light and darkness to the many other circadian oscillators in the body by providing endogenous 'zeitgebers' (Asher and Schibler 2011; Huang et al. 2011), which are more appropriately called *zeitnehmers*, since they are also both outputs and inputs of the circadian system.

The SCN's entrainment mechanism involves several *zeitnehmer* loops, on the molecular, the physiological and the behavioural level. It controls the (nocturnal)

production of melatonin but is itself responsive to melatonin (Agez et al. 2009). It also controls the daily rhythm of core body temperature. All cellular clocks, including the cellular peripheral clocks in mammalian tissue cultures, can be entrained by temperature cycles (Brown et al. 2002). The question whether temperature constitutes yet another *zeitnehmer* loop in the entrainment process of the SCN is still open. Takahashi and colleagues argue that the strong coupling of the SCN neurones makes the central pacemaker resistant to temperature changes (Mohawk and Takahashi 2011). When the authors prevented coupling between SCN neurones (by applying tetrodotoxin), one-time 6-h temperature pulses strongly reset the phase of the rhythm while they had no effect on the intact, coupled network. This result shows that isolated temperature pulses presented to a system, which has stabilised in constant conditions, may not elicit phase shifts in a robust oscillator (e.g. a strongly coupled SNC network), yet it also highlights the limitations of using pulses for explaining entrainment. As will be discussed later, the PRC concept has greatly advanced our knowledge about entrainment (Comas et al. 2006, 2007, 2008; Daan and Pittendrigh 1976) but fails to fully explain this fundamental property of circadian clocks under all conditions (Rémi et al. 2010; Roenneberg et al. 2010a, b). The ineffectiveness of a single pulse does not necessarily exclude that the SCN's neuronal network can be entrained by continuous and gradual temperature changes as they occur under normal conditions.

Abraham and co-workers (2010) have shown both conceptually (by computer modelling) and by experiments in isolated tissues that temperature cycles are indeed capable to entrain the SCN, albeit with a smaller range of entrainment than in less strongly coupled networks (e.g. lung tissue). Thus, temperature forming a *zeitnehmer* loop in the entrainment of the mammalian clock cannot be ruled out. Such a feedback loop would include all functions that can change body temperature (e.g. activity, food intake or sleep).

2 Entrainment of the Human Clock

Entrainment is also the most important property of the human circadian clock, but as will be discussed later, modern conditions of living inside and using artificial light constitute momentous challenges to human environment. First, we will review two important questions pertaining to human entrainment (1) what is the intrinsic period of the human clock (commonly used as a basis for predicting entrainment) and (2) does the human clock entrain to social cues? In addition, we show how a simple questionnaire can be used to investigate human entrainment in the real world in thousands of people.

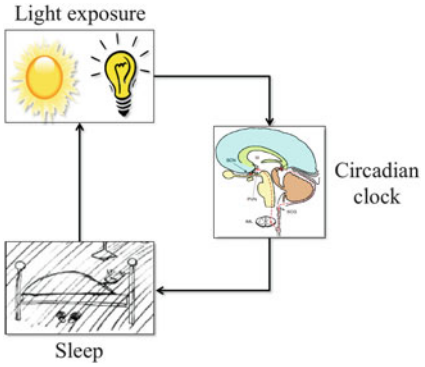

Fig. 1 Light exposure, the circadian clock and its output (the sleep–wake cycle) form a feedback loop in human entrainment

2.1 What Is the Intrinsic Period of a Circadian Clock?

Sleep itself is also an important behavioural *zeitnehmer* loop, because it influences the daily light profiles (by closing the eyelids, by retreating into a burrow or a dark room; Fig. 1). The fact that subjects in the Andechs bunker (Wever 1989) were allowed to switch off lights when they wanted to sleep has been identified as a problem for estimating the (*intrinsic*) free-running period. This self-created LD cycle prevents 'real' constant conditions, as they are thought to exist, for example, when recording rodents in constant darkness. However, the many *zeitnehmer* loops and oscillators that make up circadian systems are all an integral part of the entrainment process and may thus also influence the free-running period in constant darkness (τ_{DD}). The fact that rodents are active during their subjective night and sleep during their subjective day (with all the consequences, such as activity-/sleep-dependent temperature fluctuations or periodic food intake) makes an assessment of a 'true' *intrinsic* period questionable, even in DD because it is influenced by many other factors, for example, by the presence of a running wheel (Kuroda et al. 1997).

By making the self-sustained, free-running period a central quality/dogma of the circadian system (Pittendrigh 1960), the field has created a circular argument that has led to a selection of model organisms—namely, those which continue to show a robust rhythm in constant conditions. Theoretically, a damped clock would serve its functions perfectly in a cyclic environment, which periodically provides time cues that counteract dampening. We hypothesise that a self-sustained, free-running rhythm, measurable in constant conditions, is a consequence of a complex circadian system (including multiple oscillators and *zeitnehmer* loops). The interactions between the oscillators and the feedback provided by the *zeitnehmers* are the main reason for self-sustainment since they intrinsically provide rhythmic signals that prevent dampening. Steinlechner and colleagues have shown that the ability to free-run is challenged in clock mutants (Steinlechner et al. 2002) when they are kept in DD but not in LL. The most simple explanation for this observation is that the *zeitnehmer* feedback of the sleep–wake cycle (as shown in Fig. 1) is much

stronger when this behaviour involves modulations of light levels and therefore can turn a challenged (damped) circadian clock into a self-sustained rhythm in LL.

Circadian clocks have evolved to produce an *internal day* representing the external day. This is different to evolving a specific intrinsic free-running period (τ), for which there was no selection pressure. A steady-state τ in artificial constant conditions can only be reliably assessed when measured over several days and thus represents the average *internal day* that the circadian system produces under a given condition. τ is subject to the influence of many factors—beyond DD or LL (of different intensities)—and many of these (e.g. wheel running) will affect different *zeitnehmers* within the system and thereby change τ. While this average *internal day* (τ) is not reliable to predict entrainment under all conditions (Rémi et al. 2010), we must presume that every circadian clock produces its individual *internal day* based on genetic background. The *internal day* indeed forms the basis for entrainment, but the genetic background of an organism/individual will also influence many other aspects of the circadian machinery—from inputs via *zeitnehmers* to outputs. The difficulty of this concept is that the length of an individual clock's *internal day* cannot be measured experimentally since the entraining mechanisms will also be active in constant conditions (e.g. will be the influenced by *zeitnehmers*) and thereby change τ. Thus, the length of an *internal day* can only be assessed theoretically (see, e.g., Czeisler et al. 1999; Roenneberg et al. 2010a).

2.2 Social Zeitgebers

The notion that humans can entrain to non-photic, social cues goes back to the pioneering experiments in the Andechs 'bunker' (Wever 1979), showing that the human clock can entrain even to regular gong signals. The question whether social signals can act as zeitgebers for the human clock can best be answered by studying blind people. There are different types of blindness (1) lack of visual perception, (2) lack of residual light perception and (3) lack of physiological light responses (e.g. suppression of melatonin or pupillary reactions). While circadian rhythms in individuals of the first two types of blindness still entrain to light–dark cycles, the clocks of those, who suffer from the third type of blindness, are often not entrained [evidenced by measuring melatonin or core body temperature profiles (Sack et al. 1992)]. That their clocks run free in real life is remarkable because these individuals are submitted to strong social 24-h time cues. It suggests that the influence of non-photic zeitgebers on human clocks depends on functional light perception [even if unconscious (Zaidi et al. 2007)]. Thus, successful entrainment to non-photic time cues is apparently achieved via the behavioural *zeitnehmer* loop shown in Fig. 1 (Czeisler et al. 1986; Honma et al. 2003), indicating that the human clock does not entrain directly to social signals—otherwise blind people of all three types could successfully entrain to 24-h cycles.

Yet, how could this hypothesis explain the fact that some blind individuals of the third type—that is, without any light perception—are able to live a 24-h day (Czeisler et al. 1995; Lockley et al. 1997; Sack et al. 1992)? One possible explanation is that the length of their *internal days* is already close to 24 h. This would allow them to synchronise to relatively weak, non-photic time cues, for example, to activity-dependent temperature changes and/or to regular meals (Klerman et al. 1998; Mistlberger and Skene 2005). That non-photic signals can indeed contribute to entrainment has also been shown in the Andechs bunker experiments: the range of entrainment under LD cycles became larger when regular acoustic signals (regulating sleep–wake behaviour) were added to the protocol (Wever 1979). It follows that synchronised blind people of the third type of blindness would fail to entrain if they were exposed to schedules longer or shorter than 24 h and, conversely, that totally blind people, who do not entrain in real life, would synchronise to schedules that are closer to the length of their *internal days*.

The example of entrainment in blind individuals shows that light is also the dominant zeitgeber for human entrainment. It also suggests that entrainment may indeed involve multiple zeitgebers acting in concert, despite being insufficient—each on their own—to ensure entrainment.

2.3 Constant Versus Entrained Conditions

The fact that every organism adapts its physiology and behaviour to the alternation of day and night appeared so trivial that scientists have not seriously investigated this phenomenon until the nineteenth century [except for de Mairan (De Mairan 1729)]. The insight (and proof) that an endogenous mechanism governs the daily changes in metabolism, physiology and behaviour was only possible by experiments performed in constant conditions, showing that circadian clocks maintain a self-sustained rhythm, albeit not with an exact 24-h period.

This discovery has dominated how researchers investigate and think about circadian clocks. The focus of circadian research on free-running rhythms is overwhelming although the clock hardly ever had the chance to evolve without the presence of zeitgebers (Roenneberg and Merrow 2002). Laboratory experiments (especially those investigating the molecular mechanisms of the clock) rarely use entrainment protocols. The traditional models of entrainment assume a basic free-running period (τ) and then apply mechanisms that correct its difference to the zeitgeber period (T) by regular resets of the rhythm's phase, so that the periods of clock and zeitgeber become identical ($T - \tau = 0$). As discussed above, this assumption is correct, if based on the length of the *internal day* ($|_{E}$) but not necessarily if based on $|_{DD}$ or $|_{LL}$. Traditionally, the clock's response to light is probed experimentally by applying singular light pulses at different circadian times in DD and thereby establishing a so-called phase response curve (PRC) (Hastings and Sweeney 1958). Although PRCs have been instrumental in our understanding of entrainment (Comas et al. 2006, 2007, 2008), there are two difficulties with explaining entrainment by

singular events (e.g. a light pulse or a light–dark transition). First, it makes predictions of entrained phase in a noisy photic world extremely difficult (such predictions strictly would need to be based on PRCs generated separately for every change in intensity). Second, it puts the cart before the horse by assuming that evolution has produced an intrinsic period in constant conditions (which we have already ruled out above) and then added a mechanism to compensate for its 'inaccuracy'.

We have recently addressed the first difficulty and proposed that the circadian clock integrates light over the course of a day, which can be formally quantified by a circadian integrative response characteristic (CiRC) Roenneberg et al. 2010b). The CiRC is merely an extension of the pioneering work that leads to the establishment and perfection of the PRC (Comas et al. 2006, 2007, 2008; Daan and Pittendrigh 1976; Hastings and Sweeney 1958). But despite similar in shape to the traditional PRC, the CiRC differs in one important quality: it makes no assumptions about the mechanism that synchronises τ with T, that is, it does not presume an instantaneous response every time light levels change (phase shifts or a velocity changes). It integrates the light exposure over the past 24 h and calculates its effect on the current length of the *internal day* (τ_E). Based on the shape of PRCs, the CiRC presumes that light exposures around dawn compress and those around dusk expand the *internal day*.

In a series of experiments with the fungus *Neurospora crassa* (varying photoperiod, τ and T) (Rémi et al. 2010), we showed that only the CiRC and not the PRC can accurately predict the phase of entrainment under all applied conditions (Roenneberg et al. 2010a). The assumption that the entrainment process is based on light integration rather than on a differential detection of light changes is supported by the discovery of the circadian photoreceptor melanopsin (Freedman et al. 1999; Provencio et al. 2000). Melanopsin functions as a light integrator rather than a change detector (Lucas et al. 2003).

We still have to address the second, more fundamental difficulty. As argued above, evolution must have acted on the entrainment mechanism—genetic differences produce different CiRCs that result in individual-specific phases of entrainment, earlier or later. It follows that the observed differences in τ are a consequence rather than the basis of this genetic variability (Roenneberg and Merrow 2002). We are in the process of moving the horse back in front of the cart but still have to go a long way until we understand entrainment.

The best way to investigate entrainment is using entraining conditions, either by analysing steady-state entrainment in the laboratory (e.g. Abraham et al. 2010) or by measuring circadian properties in the real world. More recent work in mice and *Drosophila* has shown that the temporal behaviour of the classical model organisms, which have been extensively investigated in the laboratory, can be astonishingly different when measured under natural conditions (e.g. Bachleitner et al. 2007; Daan et al. 2011; Peschel and Helfrich-Forster 2011; Vanin et al. 2012).

2.4 Phase of Entrainment: Chronotype

Investigating the human clock under entrainment rather than in constant conditions has big advantages. Experiments in temporal isolation are both extremely cost- and labour-intensive (and can therefore only include few subjects). In contrast, assessing phase of entrainment by questionnaires (based on sleep times; chronotype) allows to investigate thousands of people in real life. The first instrument developed for assessing temporal sleep preferences was the morningness–eveningness questionnaire (MEQ) (Horne and Östberg 1976), which produces a score (high values indicating morning types and low values evening types). A score-based analysis is useful when chronotype is regarded as a psychological trait, and the MEQ-scores do correlate with sleep times (Zavada et al. 2005). However, when chronotype is used as a measure for entrained phase, its assessment should ideally be time- and not score-based (Roenneberg 2012). To this end, we developed a questionnaire (the Munich ChronoType Questionnaire, MCTQ), which asks simple questions about sleep behaviour separately for workdays and free days (Roenneberg et al. 2003).

Since the year 2000, the MCTQ is accessible online (http://www.theWeP.org), and the database of the ongoing MCTQ project has now exceeded 150,000 entries. Participants receive an email containing a PDF that provides individual feedback on how their results (chronotype, sleep duration, etc.) compare to those of the population stored in the database. This individualised feedback is most probably the key to the project's success. The MCTQ is available in several languages (English, German, French, Dutch, Spanish, Portuguese, Danish, Turkish, with more language variants being developed). So far, the majority of entries are from central Europe (Germany: 70 %; The Netherlands: 12 %; Switzerland 6 %; Austria: 4 %; UK 1 %; Hungary: 0.6 %; France and Italy: 0.3 %; Belgium, Spain, and Sweden: 0.2 %). In Germany, The Netherlands, Switzerland and Austria, between 0.05 and 0.08 % of the total population have filled out the MCTQ. The long-term aim of this project is to create a world-sleep-map (Fig. 2) that allows separating cultural, geographical and climatic influences from actual light entrainment.

Chronotype is assessed as the mid-phase of sleep on free days (MSF), corrected for 'oversleep' due to the sleep debt that individuals accumulate over the workweek (MSF_{sc}) (Wittmann et al. 2006). The variables of the MCTQ have been validated against sleep-logs, actigraphy, as well as cortisol and melatonin profiles measured in constant routines (Roenneberg et al. 2004 and manuscript in preparation). All these validations show highly significant correlations with chronotype assessed by the MCTQ. But does this marker represent an individual's phase of entrainment (¬)?

The internal phase relationships between different circadian outputs are not fixed. Therefore, chronotype strictly only represents Ψ of the sleep–wake cycle and not even Ψ of the activity–rest rhythm under all conditions. We have shown for example that these two rhythms respond differently to the changes in and out of daylight savings time (Kantermann et al. 2007). The internal phase relationship between the

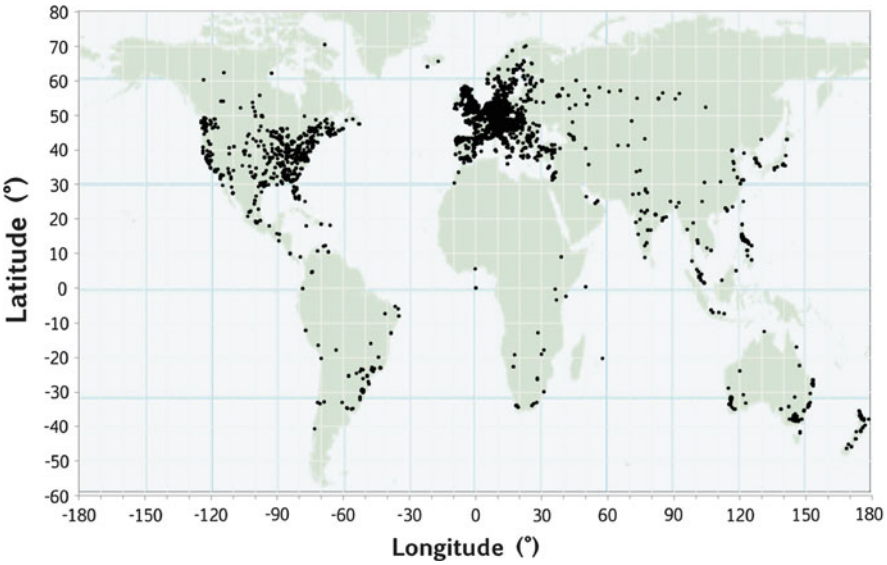

Fig. 2 Global locations of MCTQ entries. Central Europe has by far the highest representation in the database, but entries from the Americas, Asia, Oceania and to some extent from Africa are beginning to accumulate (the *dots* in the middle of oceans represent islands such as Mauritius, the Seychelles or Sao Tome). Source of the equidistant cylindrical projection of the world: http://kartoweb.itc.nl/geometrics/Map%20projections/body.htm

sleep–wake cycle and other circadian variables (e.g. melatonin or core body temperature, CBT) may vary substantially. While mid-sleep in humans is centred approximately around the time of the CBT minimum under entrained conditions, sleep is generally initiated at the time of the CBT minimum in temporal isolation (Strogatz 1987; Wever 1979). Depending on conditions, different chronotypes may also show different internal phase relationships between melatonin and sleep (Chang et al. 2009; Duffy et al. 2002; Mongrain et al. 2004).

The analysis of the growing MCTQ database has produced many important insights into human sleep–wake behaviour (for reviews, see Roenneberg and Merrow 2007; Roenneberg et al. 2007b). The most important feature of the MCTQ turned out to be the separate enquiry of sleep times on workdays and free days (see also the section on *social jetlag* below). Our analysis has clearly shown that sleep timing (chronotype) and sleep duration are separate traits. There are as many short and long sleepers among early chronotypes as there are among late chronotypes. However, when sleep behaviour is analysed separately for workdays and free days, sleep duration clearly depends on chronotype. The later their chronotype, the less sleep people get on workdays and the longer they sleep on their free days (as a compensation for the sleep debt they have accumulated during the workweek) (Roenneberg et al. 2007b).

Little is known about the mechanisms that underlie the large variability in chronotype and sleep duration. Overwhelming evidence from experiments in rodents has shown that the timing of sleep and activity depends on variants and

mutations of clock genes (see for example, Steinlechner et al. 2002). Although rodents are nocturnal and do not show the same consolidation of sleep as our species does, one can infer that human chronotype has also a genetic component (Brown et al. 2008). Indeed, several studies have shown that chronotype depends on variants of human clock genes (Jones et al. 1999; Toh et al. 2001; Xu et al. 2005). A genetic predisposition has also been shown for human sleep duration (Allebrandt et al. 2011a, b).

Besides a genetic influence, chronotype depends on several other factors, for example, on development. Children are generally early chronotypes up to the age of 14 and then significantly delay during puberty and adolescence. From the age of 20 onwards (19.5 in women and 21 in men), the entrained phase of the sleep–wake cycle is progressively advanced again until chronotype in the elderly becomes as early as in children (Roenneberg et al. 2004). The changes in chronotype between the age of 16 and 22 are often dismissed as 'typical adolescent behaviour', but Mary Carskadon argues that this change is associated with an age that used to represent the height of reproductive behaviour and that moving sleep times away from the rest of the (younger and older) population opens up a distinct temporal niche (Carskadon 2011). A recent review shows that changes in circadian timing are also found in animals, strengthening the hypothesis that this change is based on biology and not merely peer pressure (Hagenauer and Lee 2012).

Besides genes, sex and age, light exposure is another factor that determines chronotype and is subject to the following paragraph.

2.5 Light as Zeitgeber for the Human Clock

The question whether the human clock entrains to social zeitgebers or predominantly to light has been addressed above in relationship to entrainment or rather to the lack of entrainment in blind people. We have used the MCTQ database to answer this question from a different angle (Roenneberg et al. 2007a): do people within the same country live according to local time or according to the light–dark cycle? At the time of the study, the database contained approximately 40,000 German entries, including place of residence and postal code, which allowed us to reconstruct the geographical locations (Fig. 3). We then calculated the average chronotype (MSF, normalised for sleep debt, age and sex) for each longitude. Germany extends over nine latitudinal degrees, so that the sun rises 36 min earlier at the country's eastern edge than at its western edge. If the human clock entrained to social time, all Germans should have on average similar chronotypes—independent of longitude; if it however entrained to the light–dark cycle, average chronotype in each longitudinal slice should be four minutes later per longitude from east to west. The results of this 'experiment' were absolutely clear: entrainment of the human clock depends on sun time and not on local (social) time.

Although the average chronotype of people living in larger cities is later and the latitudinal slopes are flatter, chronotype still significantly correlates with sunrise.

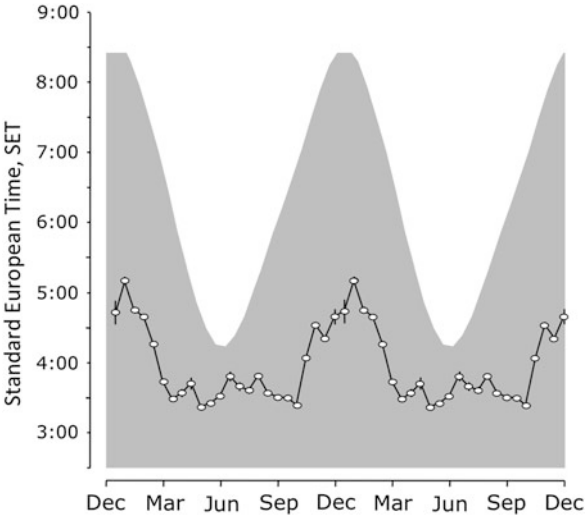

Fig. 3 Each of the locations shown on the map to the *right* represents up to several hundred entries. Their number strongly correlates with the population density of the respective location. The *horizontal axis* represents the local time of sunrise for each longitude on the longest day of the year (as reference). The *vertical axis* shows the local time of the average chronotype (MSF*sc*, normalised for age and sex) for each longitude. The stippled diagonal represents the east–west progress of sunrise. The *different symbols* represent locations with different population size; *dots*: ≤300,000; *squares*: 300,000–500,000; *triangles* >500,000 [$N \approx 40{,}000$; redrawn from Kantermann et al. (2007)]

The fact that this relationship depends on population size could be explained by different light exposure in these locations. The smaller a town, the more time people spend outdoors (e.g. access to gardens and balconies, commutes by bike or by foot) and the lower the artificial light levels at night. The greater the differences between light and darkness, the stronger the zeitgeber and the earlier the phase of entrainment (at least for the vast majority of people whose circadian clocks produce *internal days* longer than 24 h).

The MCTQ also asks how much time people spend outdoors without a roof above their heads during daylight. The analysis of this question shows that chronotype is progressively advanced the more time people spend outdoors (Fig. 4). The German latitude study has clearly shown that the human clock entrains to sunlight and not to social cues, but it did not specify which part of the light–dark cycle is most important for human entrainment (e.g. dawn or dusk). Since dawn and dusk move in opposite directions with waxing and waning photoperiod, this question can be answered by investigating the seasonality of chronotype. The results show that chronotype is aligned to dawn during winter and spring and appears to be independent of dawn or dusk during summer and autumn (Fig. 5). The fact that people are on average later chronotypes in winter is probably due to a combination of longer nights, later dawn and reduced light exposure.

The reason for these dependencies varying with season may be a combination of (1) daylight savings time (DST), of (2) the fact that locking sleep to dawn throughout the year would mean that one has to fall asleep at around 7 p.m. (local DST

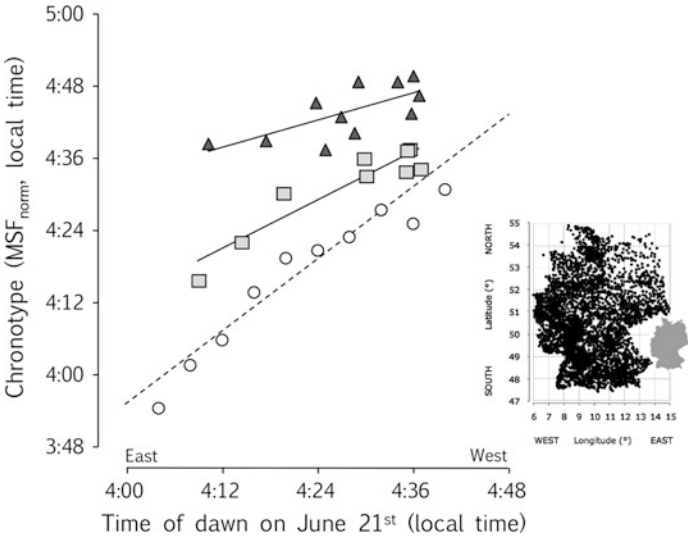

Fig. 4 With increasing time spent outdoors (during the day), the phase of entrainment advances. The strongest effects are up to an outdoor light exposure of two hours, advancing the phase by more than 2 h [$N \approx 41{,}000$; redrawn from Roenneberg and Merrow (2007)]

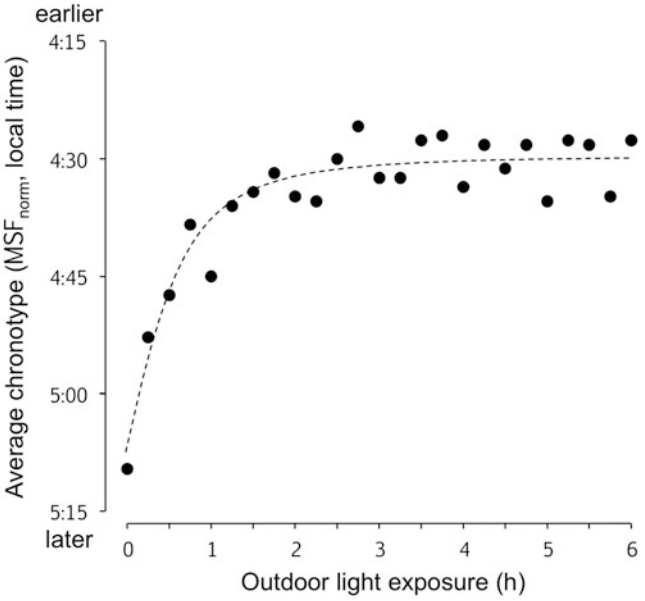

Fig. 5 Seasonal changes in phase of entrainment. *Dots* represent average chronotype (*y*-axis; expressed in Standard European Time, i.e., ignoring DST changes) across the year in half-month bins (*x*-axis). The edge of the *grey area* represents average sunrise times in Central Europe [$N \approx 55{,}000$; redrawn from Kantermann et al. (2007)]

time) in midsummer to get an average of 8 h of sleep, and (3) that in long photoperiods the time difference between dusk and dawn becomes too short, so that both factors influence phase of entrainment.

2.6 The Concept of Social Jetlag

The majority of the population represented in the MCTQ database shows large differences in sleep behaviour between workdays and free days—both in duration and timing. We have proposed that these differences represent the discrepancy *internal* and *external* time, between the control of the circadian clock and that of the social clock (predominantly set by work schedules). To quantify this phenomenon, which we have coined *social jetlag* (Wittmann et al. 2006), we calculate the difference between mid-sleep time on workdays (MSW) and on free days (MSF; Fig. 6). The example shown in Fig. 6 is extreme (a late chronotype with an early work start), but the majority of the population shows similar patterns.

Although one can sleep outside the temporal window provided by the circadian clock (e.g. naps), sleep is more efficient when coinciding with the circadian window (Wyatt et al. 1999). Around 80 % of the regularly working individuals represented in our database use alarm clocks on workdays. This premature interruption of sleep results in sleep loss (especially in the later chronotypes), because the circadian clock strongly influences when one can fall asleep. To compensate for the sleep debt accumulated over the workweek, people commonly 'oversleep' on free days (Fig. 6). While alarm clocks are the predominant cause for sleep loss in later chronotypes, social pressures to stay up later than their biological bedtime commonly causes sleep loss in early chronotypes. The majority of the Central European population in our database goes to bed at 11 p.m. or later (64 % on workdays and 90 % on free days).

Shorter habitual sleep has been shown to be associated with greater sleep debt (sleep pressure) in the sleep laboratory, indicating that interindividual habitual sleep duration primarily reflects self-selected sleep restriction (Klerman and Dijk 2005). While late types can compensate for this sleep loss on free days by 'sleeping in', early types are woken up by their circadian clock and can therefore only compensate their sleep loss by resisting the social pressure of the late majority.

The term *social jetlag* is based on the observation that sleep timing between workdays and free days resembles the situation of travelling across several time zones to the West on Friday evenings and 'flying' back on Monday mornings (Fig. 6). The symptoms of jetlag (e.g. problems in sleep, digestion and performance) are manifestations of a misaligned circadian system. In travel-induced jetlag, these complaints are transient until the circadian clock has re-entrained to the light–dark cycle at the destiny. In contrast, social jetlag is a chronic phenomenon, lasting throughout an individual's working life. 69 % of the working population represented in our MCTQ database experience at least one hour of *social jetlag* and one-third suffer from 2 h or more. Notably, the larger the discrepancy between internal and

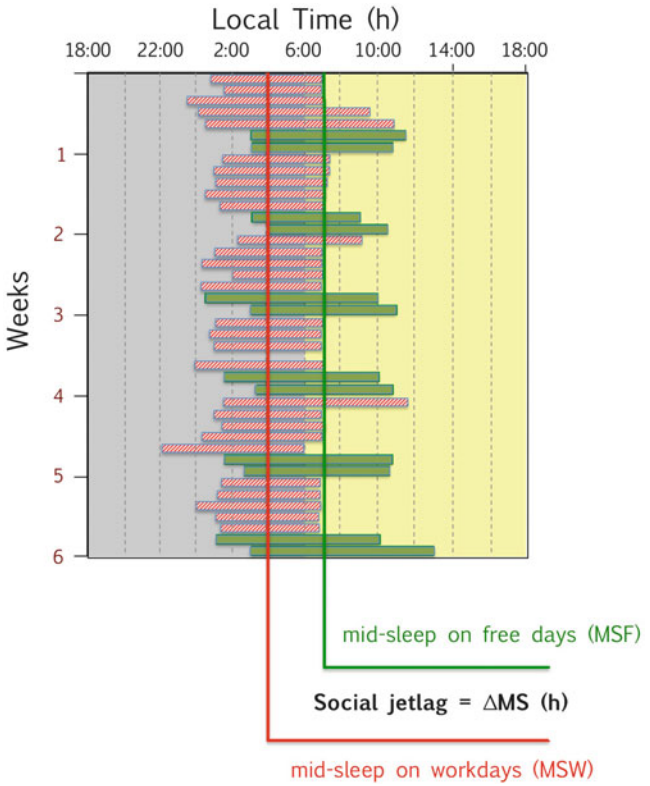

Fig. 6 Six-week long sleep-log of an extremely late chronotype (MSF ≈ 7), exemplifying the typical scalloping between sleep on workdays and on free days (*horizontal axis*: local time; *vertical axis*: days of the sleep-log. The *bars* show the timing and duration of sleep on the respective days (*red*: workdays; *green*: free days). The difference between the mid-sleep point on free days, MSF) and that on workdays (MSW) is used to quantify social jetlag. Note how sleep on workdays is interrupted by the alarm clock (constant sleep end at around 7 a.m., corresponding to internal mid-sleep of this subject)

external timing in an individual, the more likely he/she is a smoker (Wittmann et al. 2006) and the more alcohol and caffeine he/she consumes (Till Roenneberg, unpublished). In addition, every hour of social jetlag increases the chances to be overweight/obese by 30 % (Roenneberg et al. 2012).

3 Concluding Remarks

The importance of light as a zeitgeber has been well documented for virtually all plants and animals but was long debated for humans. The results of our epidemiological studies have clearly shown that the human clock entrains to light. As to be

expected for a day-active species, dawn appears to be more important than dusk for human entrainment (except in short summer nights). The fact that the correlation between 'unforced' sleep timing (e.g. on weekends) and dawn becomes flatter with growing urbanity indicates a historical change in human entrainment. In rural societies and probably throughout most of human evolution, the predominant zeitgeber was environmental light and darkness. With increasing urbanisation, which goes hand in hand with a decreasing exposure to outside light (Roenneberg et al. 2012) and an increasing self-control of the immediate light environment, human entrainment has become sleep-centric. Both sleep per se (i.e. closing our lids and rolling up our eye balls) and bedroom behaviour (retreating into darkness) are becoming the most important dark-signals that entrain the human clock (see Fig. 1). Thus, *zeitnehmers* potentially become more important to human entrainment than zeitgebers. This new situation in our evolution predicts that even sighted people, who are not solidly embedded in a social context and rarely expose themselves to the natural light–dark cycle, may be not entrained. This has already been reported for isolated cases as well as for psychiatric patients (Wulff et al. 2010).

Our epidemiological results show that social and biological time are increasingly drifting apart (*social jetlag*). The insight that sun time is more important to human temporal biology than social time has to be taken seriously by decision-makers. For example, the introduction of daylight savings time (DST), that is, making people go to work an hour earlier in summer than in winter, greatly increases *social jetlag*. Another source for *social jetlag* is the fact that work schedules have not changed significantly since our rural past, while chronotype of individuals living in industrialised regions has become too late to comply with the usual beginning of work; this has made the usage of alarm clocks reach epidemic scales. *Social jetlag* is a small but chronic version of shift-work or circadian misalignment (Scheer et al. 2009), resulting in chronic sleep restriction, substance abuse and metabolic challenges (Roenneberg et al. 2012; Wittmann et al. 2006). It is as though the majority of the population is working the early shift with all the known side effects of shift-work on health, performance and wellbeing. While 'forced desynchrony' is an important protocol in circadian laboratory experiments, one could argue that society runs a huge real-life experiment of 'forced synchrony'.

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Part IV
Systems Biology of Circadian Clocks

Mathematical Modeling in Chronobiology

G. Bordyugov, P.O. Westermark, A. Korenčič, S. Bernard, and H. Herzel

Abstract Circadian clocks are autonomous oscillators entrained by external *Zeitgebers* such as light–dark and temperature cycles. On the cellular level, rhythms are generated by negative transcriptional feedback loops. In mammals, the suprachiasmatic nucleus (SCN) in the anterior part of the hypothalamus plays the role of the central circadian pacemaker. Coupling between individual neurons in the SCN leads to precise self-sustained oscillations even in the absence of external signals. These neuronal rhythms orchestrate the phasing of circadian oscillations in peripheral organs. Altogether, the mammalian circadian system can be regarded as a network of coupled oscillators. In order to understand the dynamic complexity of these rhythms, mathematical models successfully complement experimental investigations. Here we discuss basic ideas of modeling on three different levels (1) rhythm generation in single cells by delayed negative feedbacks, (2) synchronization of cells via external stimuli or cell–cell coupling, and (3) optimization of chronotherapy.

Keywords Bifurcations • Entrainment • Modelling • Oscillations • Synchronization

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1 Introduction

The mammalian circadian clock can be regarded as a system of coupled oscillators. In virtually every cell, negative transcriptional feedback loops generate rhythm with a period of about 24 h (Zhang and Kay 2010; Minami et al. 2013; Buhr and Takahashi 2013). Circadian expression of hundreds of genes has been described in many tissues, including brain, liver, heart, and lung (Hastings et al. 2003; Keller et al. 2009; Brown and Azzi 2013). Even cultivated cells display pronounced rhythms upon stimulation (Balsalobre et al. 1998; Yagita and Okamura 2000) or temperature entrainment (Brown et al. 2002). As discussed elsewhere, the molecular clock orchestrates the timing of physiological and metabolic processes in our body (Hastings et al. 2003).

In mammals, the suprachiasmatic nucleus (SCN) is thought to play the role of the central circadian pacemaker. In the SCN, neurons are coupled via neurotransmitters and gap junctions (Welsh et al. 2010; Slat et al. 2013). The synchronization of neurons results in precise pacemaker rhythms which coordinate peripheral organs via neuronal and humoral signals. The phase of the SCN clock is entrained by external light–dark and temperature cycles. Feeding can serve as another potent *Zeitgeber*, which can entrain, for example, circadian rhythms in the liver (Stokkan et al. 2001). Circadian clock affects many physiological processes including cell division and detoxification. Consequently, the timing of therapeutic intervention can be optimized ("chronotherapy") (Lévi et al. 1997). The complexity of these processes has inspired systems biological approaches (Ukai and Ueda 2010). In particular, understanding the emergence of oscillations requires dynamical systems theory. Here, we discuss some aspects of mathematical modeling applied to circadian rhythms and chronotherapy.

Mathematical models of circadian rhythms have been applied on many levels (Pavlidis 1973; Winfree 1980; Daan and Berde 1978). Already decades ago, amplitude-phase models were developed to study entrainment properties, phase response properties, and seasonal variations (Wever 1965; Kronauer et al. 1982). Such models are still useful to study aspects of transients after jet lag (Granada and Herzel 2009), single cell oscillations (Westermark et al. 2009), effects of coupling (Bordyugov et al. 2011), and to optimize the phase response properties of circadian oscillators (Pfeuty et al. 2011). In the meantime, detailed biochemical models of the core clock have been developed (Leloup and Goldbeter 2003; Forger and Peskin 2003; Becker-Weimann et al. 2004). Such models describe transcriptional regulation, protein expression, posttranslational modifications, protein degradation, complex formation, and nuclear translocation (Relógio et al. 2011; Mirsky et al. 2009). However, quantitative details of many kinetic processes are not known and, thus, the choice of kinetic laws and parameters remains a major challenge. Simulations of coupled cells usually rely on simple cell models (Gonze et al. 2005). Recently, clock models have been connected to cell proliferation as an attempt to simulate chronotherapy (Lévi et al. 2008). Also theoretical attempts on describing systems with negative feedback and low numbers of molecules have proven the possibility of high-quality oscillations in such systems (Morelli and Jülicher 2007).

There is a statement that "all models are wrong, but some are useful" (George E. P. Box and Norman Richard Draper Wiley 1987). Indeed, mathematical models are cartoons of the overwhelming complexity of biological systems. Good models emphasize the most essential features of a system and reflect the major experimental facts. Model analysis can help to check the self-consistency of the modeling assumptions.

When the celebrated Hodgkin–Huxley model was established (Hodgkin and Huxley 1952), its theoretical analysis took as much effort as the experiments. Nowadays, computers are fast and cheap and computer simulations should complement expensive and time-consuming experiments. In many cases the development of mathematical models guides the design of appropriate quantitative measurements. In molecular chronobiology, models point to the role of transcriptional inhibition, degradation kinetics, and delays as discussed below. Mathematical models can systematically explore the role of feedback loops, the sensitivity to parameter variations and noise, and the efficacy of chronotherapies. Interesting theoretical predictions may stimulate novel experiments. Mathematical abstractions help to find common design principles of seemingly quite different biological systems. For example, most physiological oscillations are based on delayed negative feedback loops combined with cooperative interactions. Such cooperative interactions result, in turn, in a nonlinear response of the system to the feedback signal, which is required for generation of oscillations (Glass and Mackey 1988). Below we illustrate basic ideas of mathematical modeling in chronobiology using examples on different levels:

1. A simple oscillator model, which is based on a delayed negative feedback.
2. Synchronization of cells via external stimuli or cell–cell coupling.
3. Optimization of chronotherapy.

2 Oscillations Due to Delayed Negative Feedback

A large variety of physiological and biochemical oscillations has been modeled with the aid of delay differential equations (DDEs): Intracellular circadian rhythm generator (olde Scheper et al. 1999), drosophila endocycles (Zielke et al. 2011), periodic leukemia (Mackey and Glass 1977), Cheyne–Stokes respiration (Glass and Mackey 1988), blood pressure waves (Seidel and Herzel 1998), somite formation in zebra fish (Lewis 2003), circadian rhythms in *D. melanogaster* (Smolen et al. 2004), and mouse liver (Korenčič et al. 2012). Most detailed models of the mammalian clock are based on sets of ordinary differential equations (ODEs). In Appendix B, we show that DDEs and ODEs are intimately related. ODEs have been used to describe many details of the involved kinetic processes. DDEs have the advantage that fewer kinetic parameters are required. Equation (1) represents a simple DDE that can describe self-sustained oscillations:

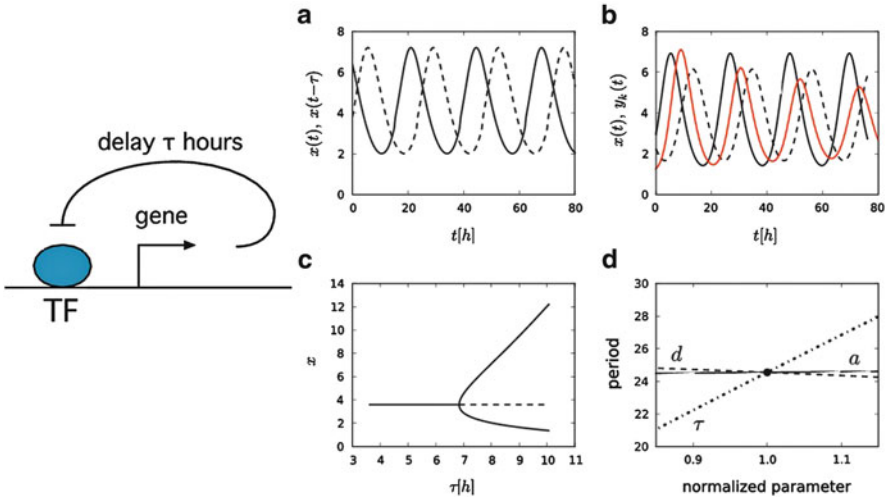

Fig. 1 *Left panel*: A sketch of a self-repressing gene regulation. Here τ denotes the time span between the transcription of the gene and its repression by its own gene products. TF represents an activating transcription factor such as BMAL1. *Right panel*: Results of simulation of Eq. (1). (**a**) A typical time course of oscillations in Eq. (1). The *solid line* denotes $x(t)$; the *dashed line* denotes the delayed variable $x(t-\tau)$. (**b**) Approximation of the DDE by an ODE system Eq. (11). *Black lines* correspond to chain length $k = 15$. The *solid black line* shows $x(k)$, and the *dashed black line* shows the time course of the last chain variable $y_{15}(t)$. The *red line* shows a decay of oscillations of $x(t)$ in Eq. (11) for chain length $k = 12$, which is not enough to successfully approximate oscillations in our DDE. (**c**) Bifurcation diagram for Eq. (1), showing a stable (the *horizontal solid line*) and unstable (*horizontal dashed line*) steady state for increasing delay τ. At $\tau \approx 7.0$ self-sustained oscillations emerge with maxima and minima shown by *solid lines*. (**d**) Dependence of oscillation period on the variation of the parameters normalized to their default values $a = 10.0$, $d = 0.2$, $\tau = 8.5$. The period is most strongly influenced by the delay τ (*dashed–dotted line*) and by the degradation rate d (*dashed line*)

$$\frac{\mathrm{d}x(t)}{\mathrm{d}t} = \frac{a}{1 + x^n(t-\tau)} - d \cdot x(t). \tag{1}$$

The dynamic variable $x(t)$ might represent a clock gene such as *Period2* whose protein product inhibits its own transcription after a delay τ (compare Fig. 1, left panel). The delay τ describes the time span between the transcription and the nuclear availability of the functional gene product. By introducing τ, we condense protein production, modification, complex formation, nuclear translocation, and epigenetic processes into a single parameter. Thus the model given by Eq. (1) is obviously a gross simplification, but it helps to understand the generation of self-sustained oscillations via delayed negative feedback loops (see Appendix A). The parameter a is the basal transcription rate, and d represents the degradation rate. A cooperativity index $n = 2$ can be justified since clock proteins frequently dimerize (Tyson et al. 1999; Bell-Pedersen et al. 2005).

For parameter values $a = 10.0$, $d = 0.2$ [a typical mRNA degradation rate (Schwanhäusser et al. 2011)], and $\tau = 8$, the model exhibits self-sustained oscillations (a "limit cycle") with a period of about 24 h. Figure 1a shows the corresponding oscillations of the state variable $x(t)$ and delayed version $x(t - \tau)$. The phase shift of 8 h resembles the phase shifts of mRNA and protein peaks of many clock genes (Reppert and Weaver 2001).

Figure 1b illustrates the close connection of DDE and ODEs. As shown in Appendix B, the explicit delay τ can be replaced by a chain of ODEs as in the widely used Goodwin model (Goodwin 1965; Griffith 1968; Ruoff et al. 2001). The corresponding auxiliary variables might represent different phosphorylation state complexes and nuclear translocation. For sufficiently long chains, ODEs approximate our DDE in Eq. (1) reasonably well.

In Appendix A, we provide a linear stability analysis of the steady state of Eq. (1). This approach allows the identification of the necessary conditions to get self-sustained oscillations. For small delays, the equilibrium is stable and perturbations decay exponentially. Intermediate delays lead to damped oscillations and further increase of τ leads to the onset of self-sustained oscillations. This transition has been termed "Hopf bifurcation" and is visualized in Fig. 1c.

The mathematical analysis in Appendix A provides further information on delayed feedback oscillators: the delay τ should be in the range of one-quarter to one-half of the period, and the inhibition should be sufficiently strong [fast decay of the transcription term in Eq. (1) with increasing "inhibitor" $x(t - \tau)$]. The period of the oscillation turns out to be nearly proportional to the delay τ.

Figure 1d displays the dependencies of the period on the model parameters τ, a, and d. As discussed above, the period grows linearly with the delay τ, whereas it decays slightly with increasing degradation parameter d. This is plausible since faster degradation implies shorter timescales of the mRNA dynamics and, hence, shorter periods. Variation of the basal transcription rate a has a minor effect on the period, consistent with Dibner et al. (2008). Extensive studies with more sophisticated models show that many insights obtained from our simple model given by Eq. (1) apply as well:

- Sufficiently strong nonlinearities are required to get self-sustained oscillations
- Overcritical delays of about a quarter to half of a period are necessary
- Delays and degradation rates have profound effects on the period

Transcriptional feedback loops with shorter-than-circadian periodicity including Hes1 (Hirata et al. 2002), p53 (Lahav et al. 2004), and NFκB (Nelson et al. 2004; Hoffmann and Baltimore 2006) have smaller delays, which result in shorter periods of a few hours. In circadian clocks, the particularly long delay is necessary to get 24 h rhythms. The central role of delays and degradation rate has been demonstrated by the intensively studied Familial Advanced Sleep Phase Syndrome (FASPS) (Vanselow et al. 2006).

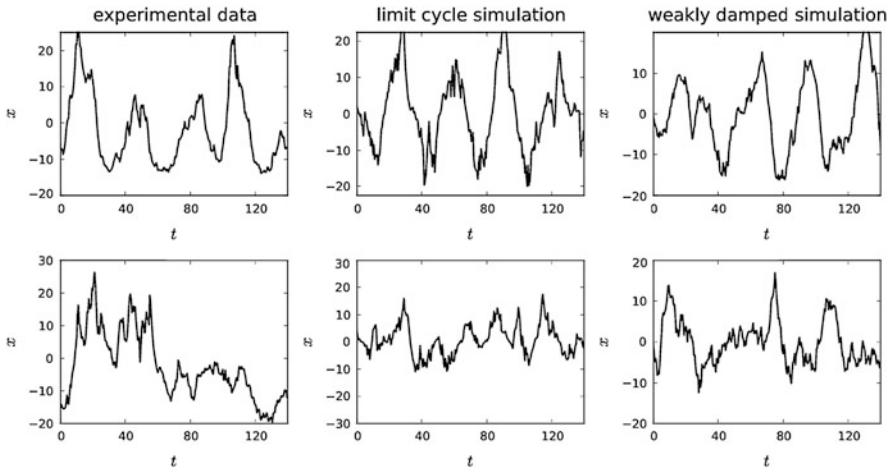

Fig. 2 Two representative bioluminescence time series from dispersed SCN neurons (*left column*). The *middle column* shows simulations of the corresponding limit cycle model (see Eq. (2)) with added noise. Similar simulations are obtained with weakly damped noise-induced oscillations (*right column*). The model parameters were estimated from the time courses in the *left-most column* as explained in (Westermark et al. 2009). This implies that the two models are tailored to the specific cells

3 Precision via Synchronization and Entrainment

On the organismic level, circadian clocks are astonishingly precise (Enright 1980; Herzog et al. 2004). Even in constant darkness (DD), the behaviorial activity onset varies from day to day by a few minutes only (Oster et al. 2002). In contrast, circadian rhythms in single cells are much noisier (Welsh et al. 1995; Liu et al. 2007), and thus for single cells, stochastic (i.e., accounting for fluctuations) models are necessary. Fitting amplitude-phase models to single cells resulted in broad distributions of estimated model parameters (Westermark et al. 2009). For example, single cell periods were found to obey a Gaussian distribution with a standard deviation of about 1.5 h (Welsh et al. 1995; Honma et al. 2004; Herzog et al. 2004). In this section, we illustrate how external signals and intercellular coupling can lead to precise circadian oscillations despite noise on the single cell scale.

We analyzed several hundred single cell recordings of circadian rhythms. The first column of Fig. 2 displays time courses of two selected cells. The upper one clearly shows periodicity, whereas the lower one is quite noisy. We have shown that such single cell data can be represented by noise-driven amplitude-phase models (Westermark et al. 2009). Interestingly, two types of fits were successful: single cell circadian time series could be modeled either by limit cycle models or as weakly damped oscillators. The examples in Fig. 2 illustrates that both types of simulations seem reasonable.

The model for self-sustained oscillations is given in polar coordinates by

$$\frac{\mathrm{d}r_i}{\mathrm{d}t} = -\lambda_i(r_i - A_i),$$
$$\frac{\mathrm{d}\varphi_i}{\mathrm{d}t} = \frac{2\pi}{\tau_i}, \quad i = 1, 2, \ldots, N. \tag{2}$$

The variable r_i is the radial coordinate, and φ_i is the phase of the i-th cell. The parameter A_i corresponds to the amplitude of the self-sustained oscillations. The parameter λ_i is the amplitude relaxation rate. Small values of λ_i result in slow amplitude relaxation towards the amplitude A_i. In order to simulate the intrinsic stochasticity of the single cell rhythms (Raser and O'Shea 2005; Raj and van Oudenaarden 2008), we added random noise to Eq. (2) (Westermark et al. 2009). Details of the simulation procedure are explained in Appendix C. Weakly damped oscillators are described by Eq. (2) with vanishing amplitudes A_i. In this way single cell rhythms can be quantified by a handful of parameters, including estimated periods τ_i and the relaxation rates λ_i.

Figure 3 shows histograms of parameters estimated from 140 dispersed SCN neurons from wild-type mice (Liu et al. 2007). The limit cycle model (left) and the damped oscillator model (right) lead to a wide range of single cell periods as reported earlier (Welsh et al. 1995; Honma et al. 2004; Herzog et al. 2004). The estimated relaxation times differ considerably between two models. Damped oscillator models exhibit smaller values of λ_i, which results in longer relaxation times. Due to slow relaxation, random perturbations can induce fairly regular noise-induced oscillations (Ebeling et al. 1983; Ko et al. 2010). Long relaxation times [or, equivalently, high oscillator qualities Q (Westermark et al. 2009)] lead to resonant behavior.

Below we discuss the response of simulated oscillators to a short pulse, external time-periodic forcing, and intercellular coupling. As parameter values, we take the direct estimates from 140 dispersed SCN neurons. We compare simulations with self-sustained oscillators and weakly damped noise-driven oscillators.

In cultivated cells, stimuli such as fresh serum, forskolin, dexamethasone, or temperature pulses can induce temporarily synchronized rhythms (Balsalobre et al. 1998; Yagita and Okamura 2000). After the stimuli are ceased, however, the synchrony is lost within a few cycles and the averaged signal damps out. This decay is caused by single cell damping and dephasing of cells due to different periods.

In Fig. 4 we compare the response of simulated cells to short pulses. For both limit cycle and weakly damped models, we observed the expected damped rhythms of the population mean. Single cells were found to exhibit much larger amplitudes than the average signal. Due to the longer relaxation time, damped oscillators (Fig. 4, right panel) show larger amplitudes and the damped oscillations persist a few more cycles. This supports our expectation that weakly damped oscillators are good "resonators." By looking only at the averaged signal (Fig. 4, time series in thick black lines), it is difficult to distinguish between limit cycle and weakly

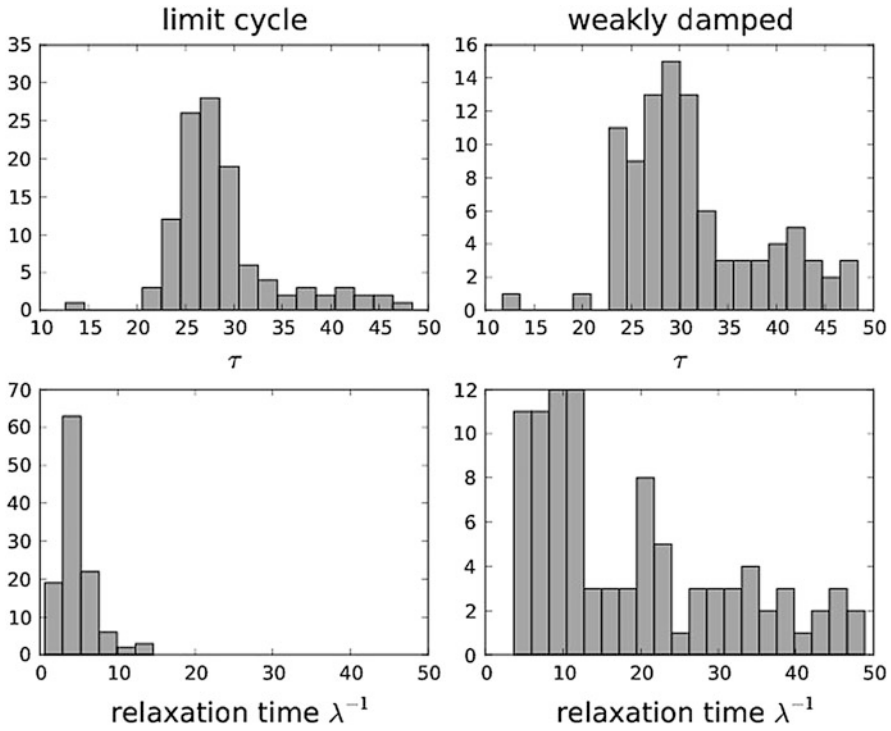

Fig. 3 Histograms of parameters estimated from a total of 140 SCN neurons. The *left column* refers to the limit cycle model (see Eq. (2)), showing a peak at circadian periods and relatively fast relaxation to their amplitudes A_i. Fitted parameters of damped oscillators (*right column*) exhibit longer relaxation times λ^{-1}, thus allowing noise-induced oscillations as shown in Fig. 2, *upper right panel*

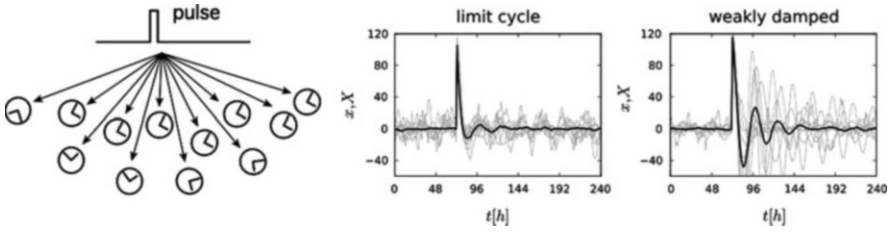

Fig. 4 Temporary synchronization of simulated cells via a pulse-like perturbation. Selected cells are visualized in *gray*. *Left panel*: Cartoon visualizing a pulse acting on oscillators. *Central panel*: Ensemble of noisy limit cycle oscillators. *Right panel*: Ensemble of noisy damped oscillators. In both time series, the *thick black line* represents the averaged signal. Parameters were extracted from experimental time series (Liu et al. 2007; Westermark et al. 2009)

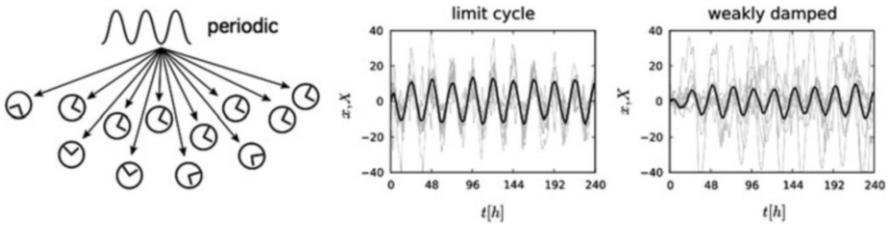

Fig. 5 Synchronization of simulated single cell oscillators by an external time-periodic *Zeitgeber* (see also Appendix C). *Left panel*: Cartoon visualizing a common periodic *Zeitgeber* acting on oscillators. *Central panel*: The averaged signal indicates that precise rhythms can be established in an ensemble of self-sustained oscillators and for weakly damped oscillators (*right panel*)

damped models. Thus, the characterization of single cell properties requires careful, long-lasting single cell experiments (Nagoshi et al. 2004; Liu et al. 2007) or resonance experiments as suggested in (Westermark et al. 2009). Such resonance experiments might be performed using temperature entrainment (Brown et al. 2002; Buhr et al. 2010). Recent studies have shown that periodic warm–cold cycles can synchronize peripheral tissues such as lung (Abraham et al. 2010) or epidermal cells (Spörl et al. 2010).

Figure 5 shows simulations of temperature entrainment. We find that relatively weak external signals can lead to fairly precise rhythms of the average signal (time series in thick black lines in Fig. 5), even though single cells are still quite noisy (time series in thin gray lines in Fig. 5). Both self-sustained oscillators (Fig. 5, central panel) and weakly damped oscillators (Fig. 5, right panel) lead to regular averaged oscillations. Some damped oscillators have relatively long relaxation time λ_i^{-1} (see Fig. 3, lower right graph), which results in large amplitudes due to stronger resonance. The distribution of the periods τ_i is however wide (see upper left plot in Fig. 3) and thus the average signal is weaker compared to the entrained self-sustained oscillator.

So far we simulated uncoupled cells synchronized via external signals. SCN neurons are coupled via gap junctions (Long et al. 2005) and neurotransmitters such as VIP (Aton et al. 2005). Such coupling leads to precise and robust SCN rhythms relatively insensitive to temperature signals (Buhr et al. 2010; Abraham et al. 2010). The periodic secretion of neurotransmitters induces a common oscillatory level, which we model by a periodic mean field, see Appendix C. Figure 6 demonstrates that such mean-field coupling can easily synchronize ensembles of noisy single cell oscillators. The coupling via mean field in Fig. 6 again leads to a pronounced amplitude expansion since a distributed neurotransmitter acts as periodic driving signal.

There is an ongoing debate whether dispersed single cells can be regarded as self-sustained oscillators or weakly damped oscillators (Nagoshi et al. 2004; Gonze et al. 2005; Westermark et al. 2009). Webb et al. (2009) find a mixture of seemingly self-sustained and damped cells the SCN. Our simulations of pulses in Fig. 4, of entrainment in Fig. 5, and of synchronization in Fig. 6 indicate that both model

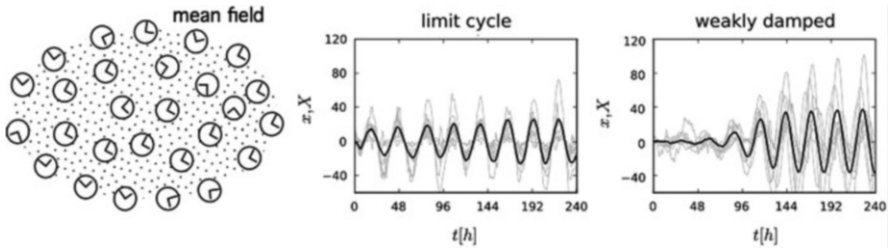

Fig. 6 Synchronization of single cell oscillators coupled through a common mean-field (*black dots* in the background of the *left panel*) as described in Appendix C. Within a few cycles coupling can induce synchrony in an ensemble of self-sustained oscillators (*central panel*) and in a set of damped oscillators (*right panel*). Single cell time-series (*thin gray lines* in the time series) reveal that selected cells exhibit quite large amplitudes due to resonance with the oscillating mean field

types can reproduce gross features of experimental observations. Consequently, long-lasting single cell recordings are needed to extract the characteristics of the oscillators. In addition, controlled resonance experiments will be helpful for determining parameters of single cell rhythms.

4 Modeling Chronotherapy

Circadian timing modifies efficacy and toxicity of many drugs (Lévi and Schibler 2007; Ortiz-Tudela et al. 2013; Musiek and FitzGerald 2013). In particular, the tolerability and efficacy of anticancer agents depend on treatment timing (Mormont and Levi 2003; Lévi et al. 1997). In mice experiments, it has been shown that a 4-h difference of drug delivery time can change the survival rate from 20 % to 80 % (Gorbacheva et al. 2005). Mathematical models of chronomodulated administration schedules ("chronotherapy") complement experiments and clinical studies (Hrushesky et al. 1989; Basdevant et al. 2005; Ballesta et al. 2011; Ortiz-Tudela et al. 2013).

Comprehensive mathematical modeling of chronotherapy should incorporate the pharmacokinetics and -dynamics (PK/PD) of drugs (Derendorf and Meibohm 1999) and the interaction of circadian rhythms and proliferation (Hunt and Sassone-Corsi 2007). Even though PK/PD models and cell cycle models (Chauhan et al. 2011) are available, the comprehensive mathematical description of chronotherapy remains an attractive challenge. Here we report the core results of recently published simulations (Bernard et al. 2010) with simple cell cycle models under periodic circadian modulation. The model is useful to study the efficacy of chronotherapeutic treatment of fast and slow growing cancer cell populations. Mathematical modeling allows simulation of various temporal treatment schedules. The central output of the model is the therapeutic index which takes into account the removal of cancer cells together with the quantification of the side-effects (Bernard et al. 2010).

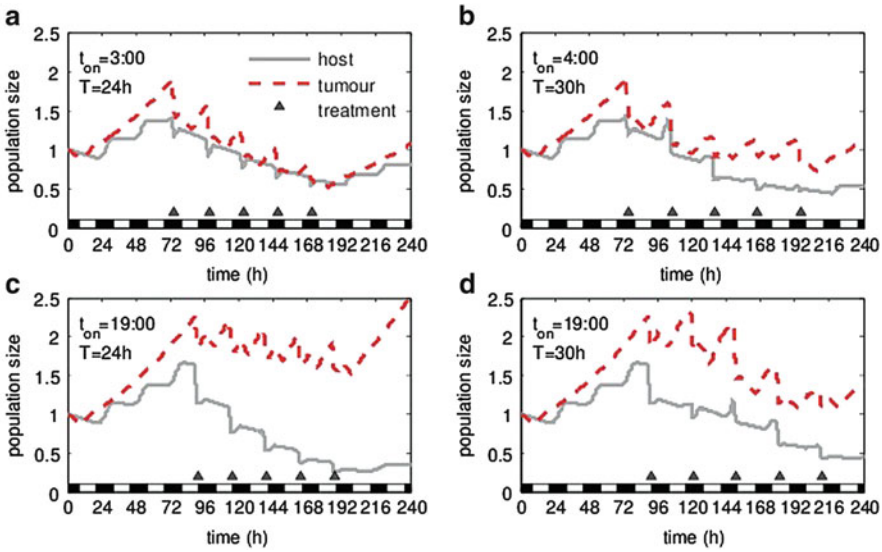

Fig. 7 Visualization of chronotherapy simulations at 24 h intervals (*left*) and 30 h intervals (*right*). The *triangles* represent treatment, *dashed lines* represent tumor growth, and *solid gray lines* host cells. The *upper graphs* show treatment at the optimal phase, whereas the *lower graphs* display the worst phases for both treatment schedules

The left graphs of Fig. 7 demonstrate the application of drugs at two different phases of a 24 h cycle. The optimal phase (Fig. 7a) and the worst phase (Fig. 7c) are compared. The simulations reproduce the experimental findings (Gorbacheva et al. 2005) that drug delivery at wrong phase can result in undesirable effects, in particular, in fast growing tumors (see Bernard et al. (2010) for details). A possible explanation for that is a resonance between the time-periodic therapy and circadian clock (Andersen and Mackey 2001). Such a resonance can be avoided by therapies with a different period. Patients often carry programmable portable pumps, and hence periods of, e.g., 30 h can be easily realized clinically. The simulation in Fig. 7d demonstrates that the 30-h-periodic treatment is successful even for the worst phase. Since it is difficult to measure the phase of circadian rhythm in a clinical situation, a treatment schedule that is applicable at any phase seems quite promising.

5 Discussion

Unfortunately, comprehensive and precise models of the mammalian circadian clock are not at the horizon. Quantitative details of many essential molecular processes such as complex formation, posttranslational modification, proteasomal

degradation, and transcriptional regulation are not available. Nevertheless, mathematical models can provide some insights into the design principles of circadian rhythms.

As shown above, simple models of delayed negative feedback loops point to the role of overcritical (i.e., beyond a certain critical value) delays and nonlinearities. For the circadian clock, there must be a minimal delay of 6 h between transcription of clock genes and their inhibition. This result emphasizes the importance of controlled degradation and nuclear translocation associated with phosphorylation and complex formation.

For many purposes traditional amplitude-phase models remain useful. For instance, phase response properties, entrainment range, and effects of coupling can be addressed with such simplified models. We have shown that temporary synchronization via pulses, entrainment, and resonance phenomena can be reproduced using amplitude-phase models with experimentally validated parameters (Westermark et al. 2009). These simulations reveal that ensembles of weakly damped single cell oscillators can constitute precise clocks thanks to coupling. This observation points to an unsolved question: How many SCN cells are in vivo truly self-sustained oscillators (Webb et al. 2009)?

In the context of optimizing chronotherapy, we simulated the interaction of circadian rhythms with cell proliferation and drug delivery. Even if many details including PK/PD were neglected, a plausible conclusion could be drawn: Due to strong resonance effects, a 24 h therapy might be more risky than other therapeutic cycles such as 30 h treatments. Of course, our minimal models have to be complemented by more detailed studies such as Ballesta et al. (2011). There are many more exciting questions that can be approached using mathematical models:

- What might be the role of auxiliary feedback loops in the core clock machinery?
- How are harmonics in gene expression profiles generated?
- How are entrainment phases controlled across seasons?
- What might be the function of the SCN heterogeneity?
- How do circadian clock, metabolism, immune response, and detoxification interact?
- What are the major selective advantages of a functioning circadian clock?

In order to address these intriguing questions, appropriate theoretical studies can successfully complement experimental approaches.

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Appendix A: Oscillations Due to Delayed Negative Feedback

A.1 The Model

One of the simplest models for a self-suppressing gene reads

$$\frac{\mathrm{d}x(t)}{\mathrm{d}t} = \frac{a}{1 + bx^n(t-\tau)} - d\,x(t). \tag{3}$$

Here, the time-dependent state variable $x(t)$ corresponds to the mRNA level of a clock gene, for instance, *Per2* at time t. The positive parameter d is the mRNA degradation rate, large values correspond to a rapid degradation, whereas small values model more stable mRNAs. Parameter a determines the basal transcription rate in the absence of the inhibitor.

The self-inhibition is modeled in the following way: For simplicity, we consciously refrain from modeling all intermediate steps, which lead from the mRNA to its protein product translocated back into the nucleus. We merely postulate that the nuclear protein abundance is proportional with the factor b to the amount of mRNA τ hours earlier. The power n is the cooperativity index, which in the case of dimerization is given by $n = 2$. The self-inhibition is reflected by the delayed state variable $x(t - \tau)$ appearing in the denominator. Its high values decrease the net production rate of the mRNA $\mathrm{d}x(t)/\mathrm{d}t$. Asymptotically, for very large values of $x(t - \tau)$, the production rate of mRNA tends to zero.

A typical choice of parameters is given by the following values: The basal transcription rate can be set to $a = 1$, because we have arbitrary units, the degradation rate to $d = 0.2\ \mathrm{h}^{-1}$, which corresponds to a typical mRNA half-life (Sharova et al. 2009), and the time delay to $\tau = 8$ h, which is a characteristic delay between *Per2* and phosphorylated nuclear PER2.

We stress that the model given by Eq. (3) is qualitative and we do not expect an exact quantitative correspondence of its predictions with the numerical values from experiments. However, many features of the oscillations can be predicted by the model equation. For example, parameter ranges of the delay τ and the degradation rate can be determined that allow the generation of oscillations.

A.2 Steady State and Its Stability

We generalize our model given by Eq. (1) to a one-dimensional DDE as follows:

$$\frac{\mathrm{d}x(t)}{\mathrm{d}t} = g(x(t-\tau)) - d \cdot x(t), \tag{4}$$

where τ is a time delay, $g(\cdot)$ is a nonlinear function, and $d > 0$ is a degradation constant. An example is the nonlinear feedback in the form of

$$g(x) = \frac{a}{1 + bx^n}, \tag{5}$$

with the parameters a, b, n as discussed above.

A steady state of Eq. (4) satisfy $\frac{\mathrm{d}x(t)}{\mathrm{d}t} = 0$ and is given by the nonlinear equation

$$g(x) - dx = 0,$$

which is in the case of Eq. (5) equivalent to

$$a - d(1 + bx^n)x = 0. \tag{6}$$

This is a nonlinear equation, which can be analytically solved only for small values of the exponent n. Generally, for arbitrary n, the steady state can be determined numerically.

Suppose that we have solved the steady state equation and the equilibrium is given by $x = x_0$. We are now interested in the question of the stability of x_0: that is, whether the system in the course of time will return back to equilibrium x_0 or depart from it. For this purpose, we introduce the ansatz

$$x(t) = x_0 + y(t), \tag{7}$$

with a small function of time $y(t)$, which is the deviation of $x(t)$ from its steady state value x_0. In order to determine the stability of x_0, we need to see, whether the derivation, $y(t)$, would grow or decay in time. We emphasize that we are interested in what happens in the intermediate neighborhood of x_0, which implies that $y(t)$ is small.

Let us introduce our ansatz into the equations. We have for the left-hand side of Eq. (4)

$$\frac{\mathrm{d}x(t)}{\mathrm{d}t} = \frac{\mathrm{d}y(t)}{\mathrm{d}t}$$

and correspondingly for its right-hand side by using a Taylor expansion up to the first order:

$$\begin{aligned} g(x(t-\tau)) - dx(t) &= g(x_0 + y(t-\tau)) - dx_0 - dy(t) \approx \\ &\approx g(x_0) + Jy(t-\tau) - dx_0 - dy(t) = \\ &= Jy(t-\tau) - dy(t). \end{aligned}$$

Here, J is the slope of the nonlinear function g in the steady state x_0 given by

$$J = \frac{\mathrm{d}}{\mathrm{d}x} g(x_0).$$

Putting both sides together results in

$$\frac{\mathrm{d}y(t)}{\mathrm{d}t} = Jy(t-\tau) - dy(t). \tag{8}$$

This is a DDE for the unknown function $y(t)$. This equation is linear, and we can solve it by an exponential ansatz

$$y(t) = y_0 \mathrm{e}^{\lambda t},$$

with the unknown complex number λ. The last ansatz, when substituted in Eq. (8), leads to

$$y_0 \lambda \mathrm{e}^{\lambda t} = J y_0 \mathrm{e}^{\lambda(t-\tau)} - d y_0 \mathrm{e}^{\lambda t},$$

and after dividing by $y_0 \mathrm{e}^{\lambda t}$ it results in the transcendental characteristic equation for λ:

$$\lambda = J\mathrm{e}^{-\lambda\tau} - d. \tag{9}$$

If we find a value λ which solves Eq. (9), the function $y(t) = y_0 \mathrm{e}^{\lambda t}$ would be a solution to Eq. (8). The growth or decay of $y(t) = y_0 \mathrm{e}^{\lambda t}$ is determined by the sign of the real part of λ. If Re $\lambda < 0$, the function $y(t)$ will decay in the course of time, which would correspond to a stable steady state x_0. If Re $\lambda > 0$, the function $y(t)$ grows, which means that the system departs from steady state x_0 and the latter is unstable.

To sum up, given steady state x_0, we have to solve Eq. (9) for the unknown λ, whose real part determines the stability of x_0. Note that Eq. (9) depends on the steady state x_0 through J, on the value of the time delay τ, and on the degradation rate d. Thus we expect that the stability of the steady state can be changed by tuning any of those parameters.

A.3 Oscillation Onset (Hopf Bifurcation)

Here, we are interested in a special situation, where the complex exponent λ has a zero real part. This corresponds to a parameter set, for which the stability of the steady state changes: if we change one of the parameters slightly, the real part will become nonzero and the steady state would either loose or gain stability, depending on the direction of the parameter change.

We introduce $\lambda = \mu + \mathrm{i}\omega$, which, when substituted in Eq. (9), results in

$$\mu = J\mathrm{e}^{-\mu\tau}\cos(\omega\tau) - d,$$
$$\omega = -J\mathrm{e}^{-\mu\tau}\sin(\omega\tau).$$

We are interested in the situation when $\mu = 0$, since it is associated with a change of stability of the steady state. At the same time when the steady state looses its stability, a small limit cycle emerges from the steady state. The period T of this limit cycle is close to $2\pi/\omega$. This scenario is known as a Hopf bifurcation. Going on with our calculations, the condition $\mu = 0$ simplifies the above two equations to

$$J\cos(\omega\tau) - d = 0,$$
$$-J\sin(\omega\tau) = \omega.$$

Using $\cos^2(\omega\tau) + \sin^2(\omega\tau) = 1$, we have

$$J^2 = d^2 + \omega^2,$$

and $d = \sqrt{J^2 - \omega^2}$. This in turn leads to the expression for the critical value of delay:

$$\cos(\omega\tau) = \frac{\sqrt{J^2 - \omega^2}}{J} = \sqrt{1 - \frac{\omega^2}{J^2}}.$$

Moreover, we can express $\omega = \sqrt{J^2 - d^2}$ and have $\cos(\omega\tau) = d/J$, which gives the value for the critical delay

$$\tau = \frac{\arccos(d/J)}{\omega} = \frac{\arccos(d/J)}{\sqrt{J^2 - d^2}}.$$

We can analyze this equation a bit further: Owing to $d \geq 0$ and $J < 0$, d/J is negative or zero. Hence, $\arccos(d/J)$ assumes values in between $\pi/2$ (corresponding to $d/J = 0$) and π (corresponding to $d/J = -1$). Thus, the value of τ is in between $2\pi/\omega$ and π/ω, which is exactly one-fourth to one-half of $T = 2\pi/\omega$. Here, T approximates the period of the limit cycle, which emerges from the steady state in a Hopf bifurcation with $\lambda = 0 + \mathrm{i}\omega$.

Our analytical calculations allowed us to specify the parameters where a Hopf bifurcation occurs: From $J = \sqrt{d^2 + \omega^2}$ we see that a certain slope is needed. Furthermore, the delay must exceed a quarter of a period (6 h for circadian rhythms). Finally, the period is approximately proportional to the delay.

Appendix B: Explicit Delays Versus Reaction Chains

In the main text we studied the DDE

$$\frac{\mathrm{d}x(t)}{\mathrm{d}t} = g(x(t-\tau)) - d \cdot x(t). \tag{10}$$

If $x(t)$ represents the mRNA of a clock gene, the transcriptional inhibition is executed by its time-delayed value $x(t-\tau)$. In reality, the mRNA is spliced, exported, and translated to a protein. The protein forms complexes, can be posttranslationally modified, and will be translocated to the nucleus, where it regulates transcription. This series of events can be modeled in principle by studying all the corresponding intermediate concentrations and the resulting inhibitory complex. Since many quantitative details are not known, we introduced here the shortcut with an explicit delay.

It turns out that variables with explicit delays can be approximated by a chain of k intermediate auxiliary variables $y_i(t)$:

$$\begin{aligned}
\frac{\mathrm{d}x(t)}{\mathrm{d}t} &= g(y_k(t)) - dx(t),\\
\frac{\mathrm{d}y_1(t)}{\mathrm{d}t} &= h(x(t) - y_1(t)),\\
\frac{\mathrm{d}y_2(t)}{\mathrm{d}t} &= h(y_1(t) - y_2(t)),\\
&\cdots\\
\frac{\mathrm{d}y_k(t)}{\mathrm{d}t} &= h(y_{k-1}(t) - y_k(t)).
\end{aligned} \tag{11}$$

If we choose $h = k/\tau$, the chain of ODEs approximates the DDE (10) [this transformation is called the *linear chain trick* (MacDonald et al. 2008; Smith 2010)]. Here we sketch a short explanation for that claim.

We begin by *ad hoc* introducing a family of gamma functions $G_{h,q}$ by

$$G_{h,q}(t) = \frac{h^q t^{q-1} \mathrm{e}^{-ht}}{(q-1)!}.$$

A first useful observation is that the time derivative of the gamma functions satisfies the following relation

$$\frac{\mathrm{d}}{\mathrm{d}t} G_{h,q}(t) = h(G_{h,q-1}(t) - G_{h,q}(t)), \quad q = 2, 3, \ldots, k,$$

which formally reminds the last k equations in (11). Using this result, a straightforward differentiation shows that functions, $y_q(t)$, given by the convolution integrals,

$$y_q(t) = \int_{-\infty}^{t} x(s) G_{h,q}(t-s)\mathrm{d}s, \quad q = 1, 2, \ldots, k, \tag{12}$$

indeed solve the last k equations in (11).

We now turn to the properties of $y_k(t)$. It is formed by convolution integrals of $x(t)$ with the gamma function $G_{h,k}(t)$. These functions have mean value at $t = k/h$ and variance proportional to k. Thus, for large k, the functions $G_{h,k}(t)$ become narrower, approximating a peak centered at k/h.

In the above integral (12) for $q = k$, we aim at localizing $x(s)$ at the time moment $t - \tau$ by properly choosing $G_{h,k}(t - s)$. The condition $s = t - \tau$ results in $G_{h,k}(t - s) = G_{h,k}(\tau)$. By an appropriate choice of parameter h, we tune the gamma function in such a way that its mean value is at the delayed time point $t - \tau$. Owing that the mean value of $G_{h,k}(\tau)$ is at $\tau = k/h$, this leads to the sought condition for h: $h = k/\tau$. We conclude that the solution of the last equation of system (11), given by

$$y_k(t) = \int_{-\infty}^{t} x(s) G_{h,k}(t - s)\, \mathrm{d}s$$

with $h = k/\tau$ indeed approximates the delayed value of x: $y_k(t) \approx x(t - \tau)$. The approximation becomes better for larger chain lengths due to narrower $G_{h,k}$ for large k.

These calculations illustrate that chains of ODEs as studied in most clock models are closely related to DDEs analyzed above. The fact that long chains (i.e., large number k of the ODE equations) lead to sharper delays could be related to the observation that many posttranslational modifications (Vanselow et al. 2006), complex formations (Zhang et al. 2009; Robles et al. 2010), and epigenetic modification (Bellet and Sassone-Corsi 2010) are involved in generation of 24 h rhythms. We also refer to Forger (2011) for a somewhat similar study of the Goodwin model as a chain of three interconnected steps.

Appendix C: Modeling Details of Single Cell Oscillators

The dynamics of single cells was described either by a noisy limit cycle model or a noise-driven weakly damped oscillator model. For N cells, the governing deterministic differential equations read:

$$\begin{aligned} \frac{\mathrm{d}r_i}{\mathrm{d}t} &= -\lambda_i (r_i - A_i), \\ \frac{\mathrm{d}\varphi_i}{\mathrm{d}t} &= \frac{2\pi}{\tau_i}, \quad i = 1, 2, \ldots, N. \end{aligned} \tag{13}$$

Here, λ_i is the radial relaxation rate, τ_i is the cell's period, and A_i is the cell's signal amplitude. All three parameters were estimated from experimental data as explained in (Westermark et al. 2009). Limit cycle oscillators have a nonzero amplitude A_i, whereas for damped oscillators we set $A_i = 0$. The cell stochasticity

was modeled by Gaussian noise sources added to the right-hand sides of Eq. (13). The variances of the noise terms were also estimated from experimental data as in (Westermark et al. 2009). For time integration of the resulting stochastic differential equation, we used the Euler–Murayama method.

For both limit cycle oscillators and weakly damped ones, three simulation protocols were realized.

- Synchronization by a pulse: At a certain time moment, we simultaneously shifted each oscillator in a specific direction by 120 dimensionless units (see Fig. 4).
- External periodic forcing: For the results presented in Fig. 5, we subjected oscillators to an external periodic force with a 24 h period and an amplitude of 0.5 dimensionless units. This driving force is much smaller than the typical oscillator amplitude of 10–20 (dimensionless units).
- Synchronization via mean field: In the third protocol (see Fig. 6), the oscillators were subjected to the mean field Z, which resulted from averaging across the ensemble:

$$Z = \frac{1}{N} \sum_{N} r_i e^{i\varphi_i}.$$

For linear damped oscillators, a saturation of the mean field at 20 dimensionless units was introduced in order to avoid amplitude explosion due to the linearity of the model.

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Mammalian Circadian Clock: The Roles of Transcriptional Repression and Delay

Yoichi Minami, Koji L. Ode, and Hiroki R. Ueda

Abstract The circadian clock is an endogenous oscillator with a 24-h period. Although delayed feedback repression was proposed to lie at the core of the clock more than 20 years ago, the mechanism for making delay in feedback repression in clock function has only been demonstrated recently. In the mammalian circadian clock, delayed feedback repression is mediated through E/E′-box, D-box, and RRE transcriptional *cis*-elements, which activate or repress each other through downstream transcriptional activators/repressors. Among these three types of *cis*-elements, transcriptional negative feedback mediated by E/E′-box plays a critical role for circadian rhythms. A recent study showed that a combination of D-box and RRE elements results in the delayed expression of *Cry1*, a potent transcriptional inhibitor of the E/E′-box. The overall interconnection of these *cis*-elements can be summarized as a combination of two oscillatory motifs: one is a simple delayed feedback repression where only an RRE represses an E/E′-box, and the other is a repressilator where each element inhibits another in turn (i.e., E/E′ box represses an RRE, an RRE represses a D-box, and a D-box represses an E/E′ box). Experimental verification of the roles of each motif as well as post-transcriptional regulation of the circadian oscillator will be the next challenges.

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1 Circadian Clock in Mammals

In mammals the master clock is located in the suprachiasmatic nucleus (SCN). Transcript analyses have indicated that circadian clocks are not restricted to SCN, but are found in several tissues including the liver (Yamazaki et al. 2000) and cultured cells such as rat fibroblasts Rat-1 (Balsalobre et al. 1998), mouse fibroblasts NIH3T3 (Tsuchiya et al. 2003), or human osteosarcoma U-2OS cells (Isojima et al. 2009; Vollmers et al. 2008). Therefore, circadian rhythms are driven by cell-autonomous oscillators. Studies across species have elucidated the conserved feature of molecular mechanisms underlying circadian rhythms: at the core of the clock lies a transcriptional/translational negative feedback loop. For example, in mice the transcription factors CLOCK and BMAL1 dimerize and activate transcription of the *Per* and *Cry* genes. PER and CRY proteins accumulate in the cytosol become phosphorylated and return to the nucleus where they inhibit the activity of CLOCK and BMAL1. The turnover of PER and CRY proteins leads to a new cycle of activation by CLOCK and BMAL1 via E/E′-box (Dunlap 1999; Griffin et al. 1999; Kume et al. 1999; Reppert and Weaver 2002; Young and Kay 2001). In this process, PER and CRY form a negative feedback loop that inhibits their own transcription. However, reciprocal activation of positive (CLOCK and BMAL1) and negative (PER and CRY) regulators in a negative feedback loop is not sufficient: there must be a delay or immediate self-inhibition of CRY and PER would result in the stable lower expression of these factors rather than oscillation. What molecular mechanism imposes this time delay? This chapter summarizes the transcription network of the mammalian circadian clock and provides insights into how the network together with post-translational regulation of clock proteins works as a delayed negative feedback loop.

2 Identification of the Circadian Transcriptional Network

2.1 Transcriptional Network Based on Three Clock-Controlled Elements

2.1.1 The E/E′-Box, the D-Box, and the RRE

The overall topology of mammalian circadian transcription network can be understood by the combination of three clock-controlled elements (CCEs), short consensus DNA sequences typically located near the promoter region of clock genes. These CCEs are called the E/E′-box (CACGT(T/G)) (Gekakis et al. 1998; Hogenesch et al. 1997; Ueda et al. 2005; Yoo et al. 2005), the D-box (DBP response

element) (TTATG(C/T)AA) (Falvey et al. 1996; Ueda et al. 2005), and the RRE [RevErbA response element, also called as ROR response element (RORE)] [(A/T) A(A/T)NT(A/G)GGTCA] (Harding and Lazar 1993; Preitner et al. 2002; Ueda et al. 2002, 2005).

By performing transcriptome analysis, expression of 24-h periodic genes was reported in cultured cells (Grundschober et al. 2001), the SCN (Panda et al. 2002; Ueda et al. 2002), and other tissues such as heart (Storch et al. 2002), liver (Panda et al. 2002; Storch et al. 2002; Ueda et al. 2002), aorta (Rudic et al. 2005), adipose tissues (Zvonic et al. 2006), calvarial bone (Zvonic et al. 2007), and hair follicle (Akashi et al. 2010). Although there are differences in the rhythmicity of circadian-expressed genes in each tissue, the following mammalian clock genes most commonly have circadian oscillation: *Period1* (*Per1*), *Per2*, *Per3*, *Dec1* (*Bhlhb2*), *Dec2* (*Bhlhb3*), *Cryptochome1* (*Cry1*), *Clock*, *Npas2*, *Bmal1* (*Arntl*), *Dbp*, *E4bp4* (*Nfil3*), *RevErbAa* (*Nr1d1*), *RevErbAb* (*Nr1d2*), and *Rora*. The temporal expression of each gene is controlled by a different combination of CCEs. Evolutionary conserved E/E′-boxes are located in the noncoding regions of nine genes (*Per1*, *Per2*, *Cry1*, *Dbp*, *Rorγ*, *RevErbAa*, *RevErbAb*, *Dec1*, and *Dec2*), D-boxes are contained in eight genes (*Per1*, *Per2*, *Per3*, *Cry1*, *RevErbAa*, *RevErbAb*, *Rorα*, and *Rorβ*), and RREs in six genes (*Bmal1*, *Clock*, *Npas2*, *Cry1*, *E4bp4*, and *Rorc*). The expressed gene product positively or negatively regulates transcription activity by acting on CCEs: CCEs and these clock genes form a closed network structure (Fig. 1) as described below.

2.1.2 Transcription Regulation via the E/E′-Box and Clock Genes

The E/E′-box is positively regulated by *Bmal1*, *Clock*, and *Npas2* and negatively regulated by *Per1–3*, *Cry1–2*, and *Dec1–2*. CRY and PER are hypothesized to autoregulate their own expression by repressing the heterodimeric complex of the basic helix–loop–helix (bHLH) PER-ARNT-SIM (PAS) domain transcriptional activators CLOCK and BMAL1, which bind to E/E′-box elements in the *Cry1* and *Per1–2* promoters. Although both positive regulators (*Bmal1*, *Npas2*, *Clock*) and negative regulators (*Per1–3* and *Cry1–2*) have circadian rhythmic expression patterns, peak time of positive regulators are antiphase to that of negative regulators (delayed negative feedback).

2.1.3 Transcription Regulation via the D-Box and Clock Genes

The D-box is positively regulated by PAR-bZIP (proline- and acidic amino acid-rich basic leucine zipper) transcription factors (*Dbp*, *Tef*, and *Hlf*) and negatively by *E4bp4*. Like the E/E′-box, an antiphase relationship of gene expression between negative and positive regulators can be observed. In the D-box case,

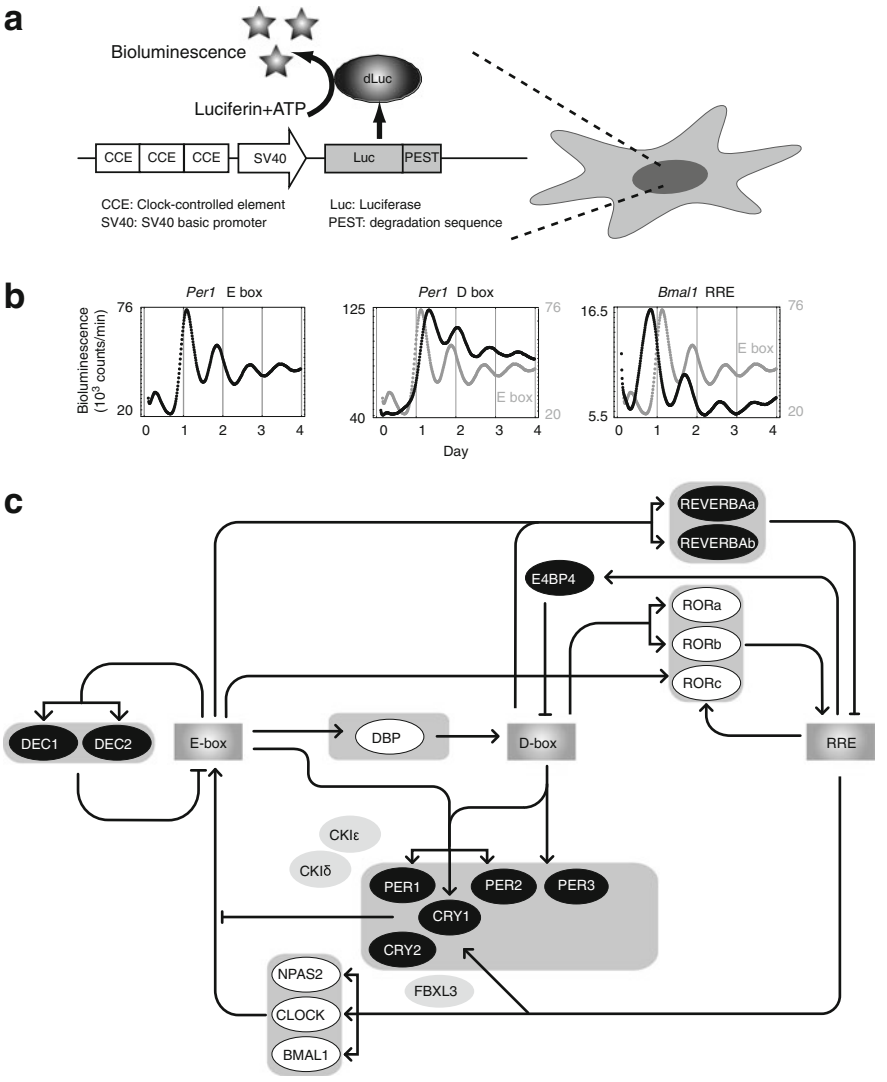

Fig. 1 Schematic representation of the transcriptional network of the mammalian circadian clock. (**a**) In vitro cycling assay. Cultured mammalian cells (Rat-1) were transfected with d*Luc* under the control of a clock-controlled element (CCE) and SV40 basic promoter (Ueda et al. 2005). (**b**) Representative circadian rhythms of bioluminescence from a wild-type *Per1* E-box CCE fused to the SV40 basic promoter driving a d*Luc* reporter (*left panel*) and compared to bioluminescence rhythms driven by a *Per1* D-box (*center panel*) and a RRE (*right panel*). Original figures are reproduced from Ueda et al. (2005). (**c**) Genes and CCEs are depicted as *ellipsoids* and *rectangles*, respectively. Transcriptional/translational activation is shown by *arrows* (→) and repression is depicted by *arrows with flat ends* (⊣)

the expression phase of the positive regulator *Dbp* is similar to that of *Per1*, whereas the expression phase of the negative regulator *E4bp4* is similar to that of *Bmal1* (Mitsui et al. 2001).

2.1.4 Transcription Regulation via the RRE and Clock Genes

RRE is positively regulated by *Rora*, *Rorb*, and *Rorc* and negatively regulated by *RevErbAa* and *RevErbAb*. In the SCN, *Rora* and *Rorb* have circadian rhythms but not *Rorc* (Ueda et al. 2002). Liu et al. reported that *RevErbAa* and *RevErbAb* are functionally redundant and necessary for oscillation of the RRE-regulated gene *Bmal1*. By contrast, *Ror*s contribute to *Bmal1* amplitude, but are not required for generating oscillation (Liu et al. 2008).

2.1.5 Timing of Each CCE

The circadian timing at which each element becomes active for transcription can be monitored with an in vitro cell culture system in which a destabilized firefly luciferase (d*Luc*) reporter is driven by different clock-controlled promoters. After cells are synchronized (i.e., with dexamethasone, forskolin, or serum), oscillations in gene expression are recorded by bioluminescence (Nagoshi et al. 2004; Ueda et al. 2002, 2005; Welsh et al. 2004). Using this in vitro cycling assay, the "phase" of each CCE can be determined (Ueda et al. 2002, 2005) (Fig. 1). Note that the term "phase" used in this chapter represents relative peak timing of each circadian gene expression within single circadian cycle. Each CCE is responsible for the gene expression at distinct circadian times: the peak time of E/E′-box-driven expression is followed by D-box-driven expression after an interval of ~5 h. Then, RRE-driven expression follows D-box expression after ~8 h. E/E′-box-driven expression begins to appear again ~11 h after RRE-driven expression. In the case of the SCN, the subjective time drawn by each CCE can be illustrated as "morning-time" for the E/E′-box, "evening-time" for the D-box, and "nighttime" for the RRE (Ueda et al. 2005).

2.2 Importance of Gene Regulation via the E/E′-Box

2.2.1 Circadian Clock Perturbation via CCEs

The three CCEs have different impacts on cellular circadian rhythms: perturbation of E/E′-box regulation abolishes circadian rhythms; perturbation of RRE regulation has an intermediate but significant effect; and D-box disruption has almost no effect.

A study using Rat-1 cell showed this by overexpressing regulatory genes with repressive activity to different CCEs (Ueda et al. 2005) (Fig. 2). The *Per2* promoter is regulated via an E/E′-box and a D-box, and the *Bmal1* promoter is regulated via

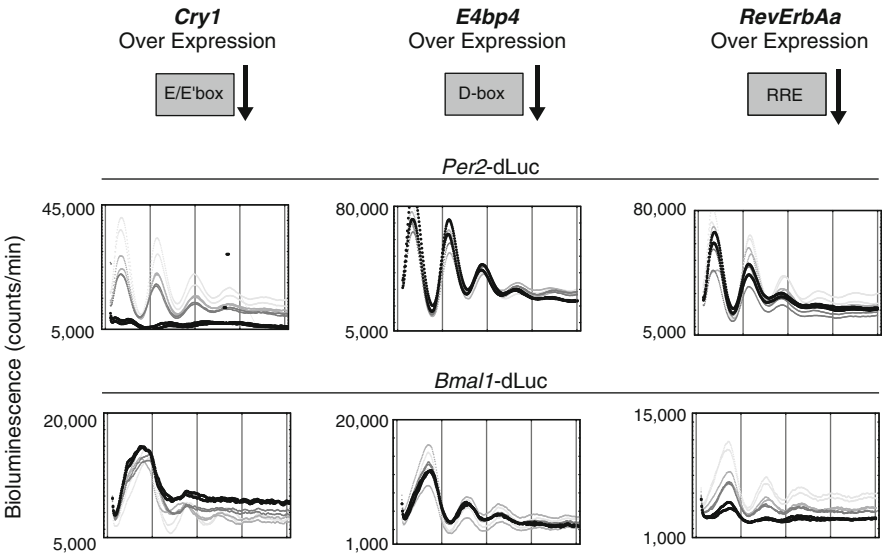

Fig. 2 Importance of the E/E′-box. Effect of repression on each CCE. The E/E′-boxes, D-box, and RRE were repressed by overproduction of CRY1, E4BP4, and REVERBAa, respectively. The consequences of those repressions were monitored by bioluminescence from *Per2* and *Bmal1* promoter driving a destabilized luciferase (*Per2*-dLuc and *Bmail1*-dLuc). Original figures are reproduced from Ueda et al. (2005). The *different shades of gray* in the plot indicate different amounts of transfected vector

an RRE. When E/E′-box activity is perturbed by overexpression of the *Cry1* gene, both *Per2*-promoter-driven reporter gene (*Per2*-dLuc) and *Bmal1*-promoter-driven reporter gene (*Bmal1*-dLuc) lose circadian rhythms. When an RRE is perturbed through *RevErbAa* overexpression, *Bmal1*-dLuc loses circadian rhythms and the amplitude of *Per2*-dLuc rhythmic expression is decreased. The impact of RRE perturbation through *RevErbAa* overexpression appeared to be more significant in mice liver. Kornmann et al. showed that liver-specific overexpression of *RevErbAa* abolishes the rhythmicity of PER2::Luc expression in liver explants (Kornmann et al. 2007). Contrary to the case of E/E′-box and RRE, D-box perturbation through *E4bp4* overexpression causes both *Per2*-dLuc and *Bmal1*-dLuc transcriptional activity to have normal circadian rhythms (Ueda et al. 2005). These varying effects are difficult to explain by mere quantitative differences in the strength of the three repressors, which suggests that there is some qualitative difference between E/E′-box, D-box, and RRE regulation in circadian rhythmicity.

2.2.2 Circadian Feedback Repression: Heart of the Circadian Transcriptional Network

PER and CRY play key roles in the circadian clock transcriptional network by closing the negative feedback loop of E/E′-box regulation. CRY has stronger

repressor activity than PER (Kume et al. 1999). Sato et al. reported that interference of CRY1's repressor activity on E/E′-box-mediated transcription can abolish circadian transcriptional oscillations. They screened both human CLOCK and BMAL1 alleles that were insensitive to CRY1 repression but maintained normal transcriptional activity. Selected clones have normal transcriptional activities similar to wild type in the absence of CRY1, but have greater reporter activity in the presence of CRY1. By analyzing either *Per2*-dLuc or *Bmal1*-dLuc, they observed that cotransfection of either CLOCK or BMAL1 mutant alleles resulted in substantial impairment of circadian rhythmicity after one or two cycles of oscillation; cotransfection of both CRY-insensitive mutant CLOCK and BMAL1 together resulted in the loss of circadian promoter activity. This suggests that transcriptional repression of CLOCK/BMAL1 by CRY1 is required for circadian regulation via both an E/E′-box and an RRE (Sato et al. 2006) (Fig. 3).

3 Minimal Circuit of the Mammalian Circadian Clock

3.1 Two Delayed Negative Feedback Loops

How is the negative feedback to an E/E′-box delayed? Although there is an E′-box and an E-box in *Cry1*'s regulatory region (Fustin et al. 2009; Ueda et al. 2005), the peak of *Cry1* expression is evening-time, which is substantially delayed relative to other genes with an E/E′-box (Fustin et al. 2009; Ueda et al. 2005). *Cry1* has two functional RREs in one of its introns (Ueda et al. 2005) and also D-box in its promoter region (Ukai-Tadenuma et al. 2011). Ukai-Tadenuma et al. experimentally confirmed that the combination of daytime elements (D-box) and nighttime elements (RREs) within its intronic enhancer gives rise to *Cry1*'s delayed evening-time expression. Interestingly, the observed delayed expression was well explained by a simple phase-vector model that enabled artificially designed delayed expressions (Ukai-Tadenuma et al. 2011) (Fig. 4).

Based on this simple phase-vector model (Fig. 4), they generated an array of *Cry1* constructs that have different phases and used these in a genetic complementation assay to restore circadian oscillation in arrhythmic $Cry1^{-/-}$:$Cry2^{-/-}$ cells established from $Cry1^{-/-}$:$Cry2^{-/-}$ double-knockout mice (van der Horst et al. 1999). These experiments reveal that substantial delay of *Cry1* expression is required to restore single-cell-level rhythmicity and that prolonged delay of *Cry1* expression can slow circadian oscillations (Fig. 5). These results suggest that phase delay in *Cry1* transcription is required for mammalian clock function and these results provide formal proof that the design principle of the mammalian circadian clock transcriptional network is negative feedback with delay (Ukai-Tadenuma et al. 2011).

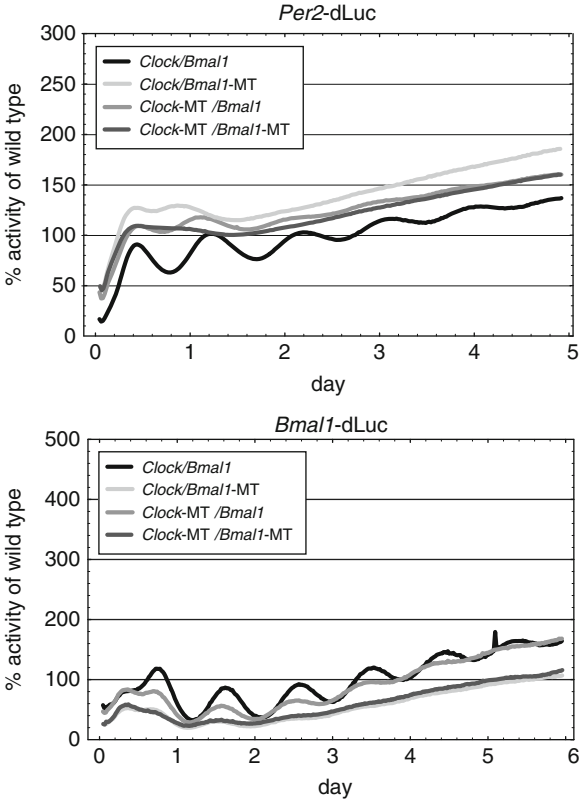

Fig. 3 The impairment of CRY-mediated repression. Coexpression of CLOCK/BMAL1 mutant heterodimers that are insensitive to CRY repression ablates circadian E-box and RRE activities in NIH3T3 cells. Plasmids expressing Flag-tagged CLOCK and BMAL1 alleles were transiently cotransfected with the *Per2*-dLuc (*upper panel*) or *Bmal1*-dLuc reporter plasmid into NIH3T3 cells (*lower panel*). *Per2* or *Bmal1* promoter activities in NIH3T3 cells transfected with single or double CRY1-insensitive CLOCK, and BMAL1 mutants (MT) were monitored over 5 (*upper panel*) or 6 days (*lower panel*). All reporter activities were normalized such that the median wild-type luciferase activity over the time course was 100 %. Original figures are reproduced from Sato et al. (2006)

Based on these results, they hypothesized that the transcriptional network can be simplified into a model consisting of two transcriptional activations and four transcriptional repressions on three regulatory DNA elements (Fig. 6). Notably, this diagram can be envisaged as a composite of two distinct oscillatory network motifs (1) a repressilator, which is composed of three repressions, and (2) a delayed negative feedback loop, which is composed of two activations and one repression. Both oscillatory network motifs include delayed feedback repression and can generate autonomous oscillations independently (Elowitz and Leibler 2000; Stricker et al. 2008).

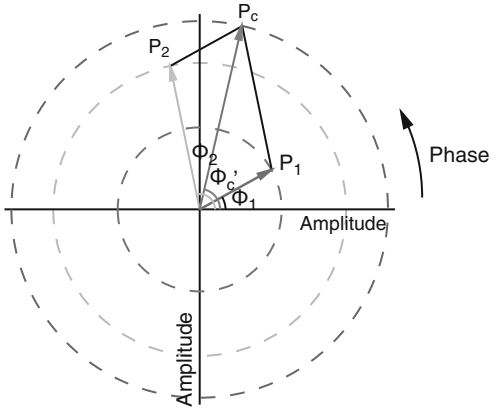

Fig. 4 Phase-vector model. A new phase results from the combinatorial synthesis of two transcriptional regulators or two clock-controlled DNA elements, which can be illustrated to a first-order approximation by a phase-vector model. This combinatorial regulatory mechanism for generating new circadian phases of transcription represents a general design principle underpinning complex system behavior. Assume wave function $f_x(t) = A_x \cos(\theta(t) + \phi_x)$. The amplitude of wave A is represented by the length of a phase vector P, and the phase of wave ϕ is represented by the angle of P. The component waves f_1 and f_2 are displayed by phase vectors P_1 and P_2. P_c is the summed phase vector of P_1 and P_2. Original graph is reproduced from Ukai-Tadenuma et al. (2011)

3.2 Genetic Evidence for the Importance of CCEs

The minimal circuit model implies that all of three CCEs have substantial importance for the circadian oscillator. The importance of an E/E′-box-mediated regulation is manifested by the phenotypes of several clock gene-knockout mice. The circadian clock governs physiological phenomena like day–night variation of activity, so changes in behavioral rhythms reflect differences in the endogenous clock of mutant mice. Accordingly, disruption of *Bmal1*, a positive regulator of E/E′-box-mediated regulation, directly results in the loss behavioral rhythms in mice (Bunger et al. 2000; Shi et al. 2010). Disruption of *Clock* gene did not result in loss of behavioral rhythms (DeBruyne et al. 2007) probably because *Clock* and another gene *Npas2* have redundant roles: *Clock* and *Npas2* double-knockout mice have arrhythmic behavioral patterns (DeBruyne et al. 2007), while *Npas2*-disrupted mice have normal behavioral rhythms (Dudley et al. 2003). Loss of negative regulator of E/E′-box-mediated transcription also results in arrhythmic phenotypes. Both *Per1* and *Per2* disrupted mice have loss of circadian rhythmicity of behavioral activity (Bae et al. 2001; Zheng et al. 2001), and $Cry1^{-/-}$:$Cry2^{-/-}$ mice have arrhythmic behavioral patterns (van der Horst et al. 1999; Vitaterna et al. 1999).

The minimal structure shown in Fig. 6 implies that D-box and RRE also play an essential role to maintain the delay time for negative feedback. For example, the double knockout of *RevErbAa* and *RevErbAb* mice has arrhythmic

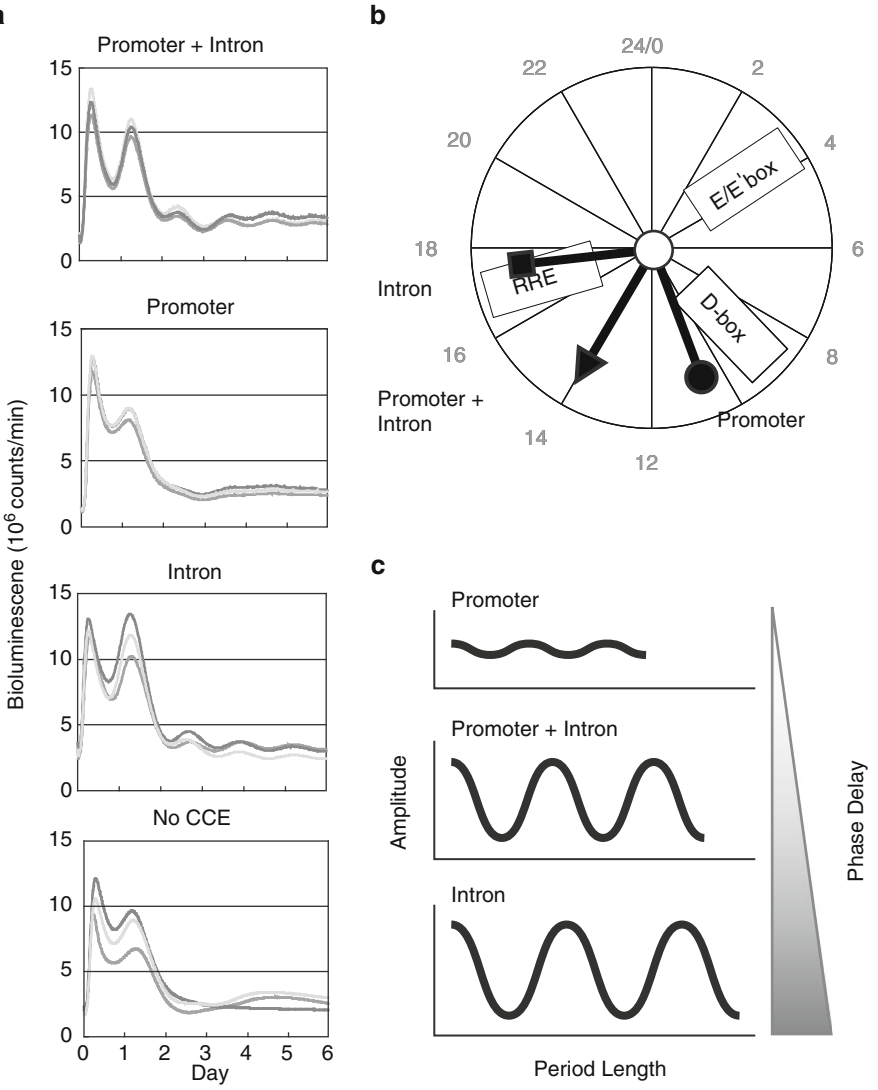

Fig. 5 The biological relevance of delayed *Cry1* expression in circadian clock function. (**a**) *Per2*-dLuc bioluminescence levels in transfected *Cry1*$^{-/-}$:*Cry2*$^{-/-}$ cells. The *Per2*-dLuc reporter and a *Cry1* expression construct were cotransfected into *Cry1*$^{-/-}$:*Cry2*$^{-/-}$ cells. (**b**) *Cry1* expression phases under different promoters (Ueda et al. 2005; Ukai-Tadenuma et al. 2011) that contain either a D-box (*Cry1* promoter), RRE (*Cry1* intron), or both (promoter + intron). (**c**) Substantial delay in feedback repression is required for mammalian clock function. The decreased delay dampens the amplitude of circadian oscillations (*top panel*), and the prolonged delay in feedback repression slows the frequency of circadian oscillations (*bottom panel*) compared to wild type (*middle panel*). Original figures are reproduced from Ukai-Tadenuma et al. (2011). *Different trace shades* represent results from triplicated experiments

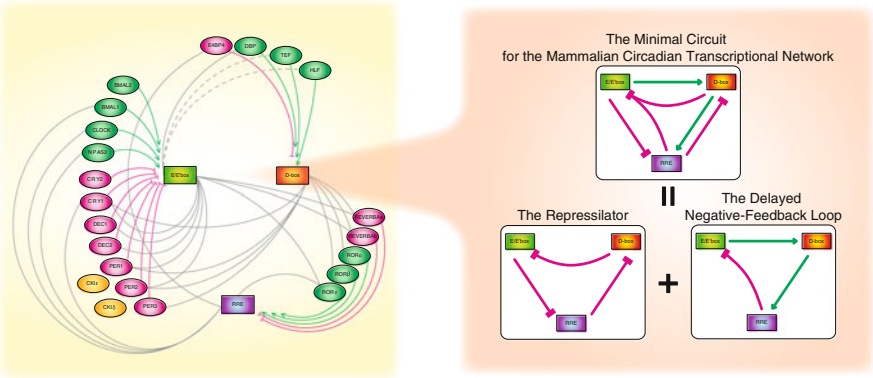

Fig. 6 The minimal circuit for the mammalian circadian transcriptional network. (**a**) The transcription network of the mammalian circadian clock (Ueda et al. 2005; Ukai-Tadenuma et al. 2011). (**b**) The minimal circuit (*top panel*) can be illustrated as a composite of two distinct oscillatory network motifs: a repressilator (*bottom left panel*) and a delayed negative feedback loop (*bottom right panel*). Transcriptional activation (*arrows*); transcriptional repression (*arrows with flat ends*); regulatory DNA elements (*rectangles*; E/E′-box, morning; D-box, daytime; RRE, nighttime). Original graph is reproduced from Ukai-Tadenuma et al. (2011)

behavioral phenotypes and arrhythmic clock gene expression (Bugge et al. 2012; Cho et al. 2012).

The importance of D-box transcriptional regulators is still unclear because no report shows that dysfunctional mice for D-box regulators have completely arrhythmic behavioral patterns. Lopez-Molina et al. reported that *Dbp* knockout mice have normal behavioral rhythms compared to wild type (Lopez-Molina et al. 1997). *Hlf* or *Tef* disrupted mice also have almost normal behavioral rhythms (Gachon et al. 2004). Even triple knockout of PAR-bZIP transcriptional factor mice have almost normal behavior rhythms (Gachon et al. 2004). Although *E4bp4* knockout mice was constructed (Gascoyne et al. 2009), behavioral rhythms of the mice were not reported.

3.3 Generation of Various Phases by the Combination of CCEs

From DNA microarray data, more than 10 % of expressed genes have circadian rhythms with a wide range of peak timings (Delaunay and Laudet 2002); the distribution of peak timing is not limited to three circadian times corresponding to the expression timing of each CCE. How do these "intermediate" expression timings arise? One possibility is that the combination of three CCEs generates various circadian phases.

Ukai-Tadenuma, Kasukawa et al. adopted a synthetic approach to physically simulate the correlation between CCEs combinations and the peak timing of

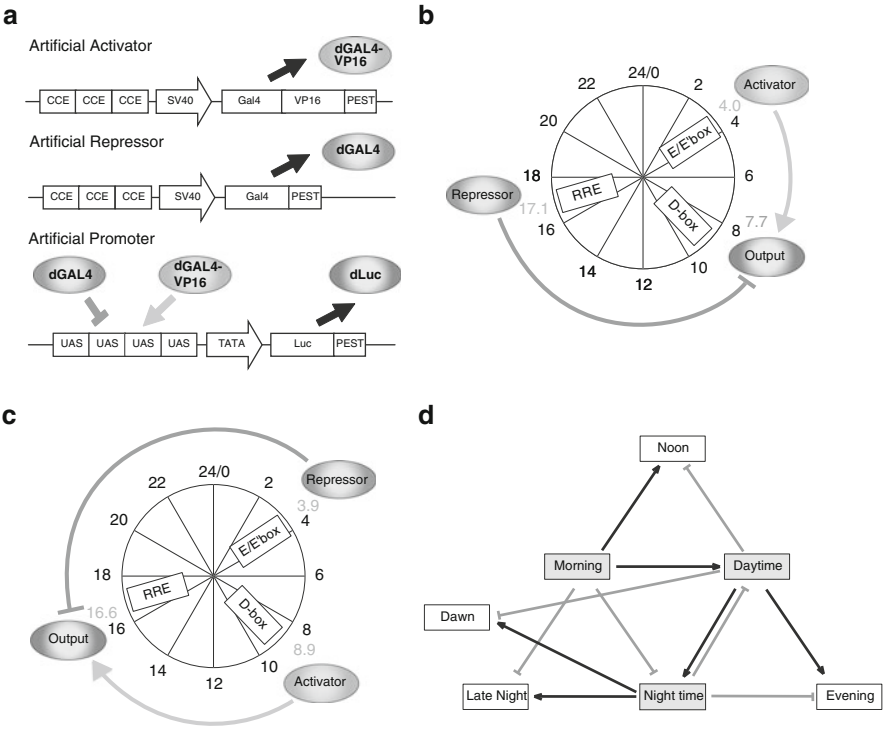

Fig. 7 Combinatorial regulation of circadian phases by a synthetic system. (**a**) The artificial transcription system. Activator and repressor are driven under clock-controlled elements (CCEs). Details are described in main text. (**b**, **c**) Promoter activities of an activator, repressor, and output in different artificial transcriptional circuits. The schemes summarize the representative promoter activities of each artificial circuit monitored by bioluminescence from NIH3T3 cells, where an activator, repressor, and output phases are indicated with their peak time (*gray numbers*). Morning activator under E′-box control and nighttime repressor under RRE control and (**b**) daytime activator under D-box control and morning repressor under E′-box control (**c**). (**d**) The relationship of the expression timings of the transcription factors and output. Various expression timing is generated from three basic phases (morning, daytime, and nighttime). *Black lines* indicate activation (*arrows*) and *gray lines* repression (*arrows with flat ends*). Original figures and graphs are reproduced from Ukai-Tadenuma et al. (2008)

expression. They used three components: an artificial activator (dGAL4-VP16), an artificial repressor (dGAL4), and a dGAL4-VP16-driven reporter gene (d*Luc*) as an output (Fig. 7a). If the expression of artificial activator and repressor are controlled by different CCEs, then the output may vary according to a combination of the various peak timings of each CCE. By taking the peak expression timing of clock gene expression in mouse liver, phase of each CCE-driven gene expression can be related with subjective circadian time: E/E′-box-driven expression peak timing as "morning," RRE-driven expression peak timing as "night," and D-box-driven peak timing as "daytime." They created "daytime" expression by the combination of E/E′-box (morning)-driven activator and RRE (night)-driven repressor (Fig. 7b). This

is similar to transcriptional regulation via D-box control; D-box is activated by E/E′-box-controlled *Dbp* and repressed by RRE-controlled *E4bp4*, and output phase is "daytime." Next, they created "night" by the combination of a D-box-driven activator and an E/E′-box-driven repressor (Fig. 7c). This is similar to an RRE with output phase "night": RRE is regulated by D-box-driven activator (*Rora*) and E/E′-box-driven repressor (*RevErbAa*), though *RevErbAa* is also controlled by D-box. By combining these CCEs in different arrangements, Ukai-Tadenuma, Kasukawa et al. also generated additional phases (Fig. 7d), which are not identical to any of the original CCE timings (Ukai-Tadenuma et al. 2008).

4 Post-Translational Regulation, Another Layer of Delay or Another Oscillator?

4.1 Phosphorylation of PER

As we discussed above, accumulating evidence indicates that Cry1-mediated delayed negative feedback plays a critical role in the circadian transcription network. If so, is the network structure of transcription activator/inhibitor relationship sufficient for generating mammalian circadian properties? If we replace all transcription factors with artificial ones [such as GAL4-VP16 used in Ukai-Tadenuma et al. (2008)] but keep the network structure, could we reproduce a robust circadian system? Natural circadian systems, however, seem to be more complex than the transcription-translation network; post-translational regulation is also critical for circadian function (Gallego and Virshup 2007). In particular, phosphorylation of PERs by CKIδ/ε is one of the determinants of circadian period length (Lowrey et al. 2000; Toh et al., 2001; Xu et al., 2005). The first circadian mutant identified in mammal was the *tau*-mutant hamster, which has a shorter behavioral period length compared to a normal hamster (Ralph and Menaker 1988). Takahashi's group identified the *tau* mutation in the *CKIe* gene and found that PER phosphorylation is lower in *tau*-mutant hamsters (Lowrey et al. 2000). The importance of PER phosphorylation by CKIδ/ε for circadian rhythms is also true in humans. Toh et al. discovered that familial advanced sleep-phase syndrome (FASPS) is caused by a mutation in the CKIδ/ε binding site of PER2 (Toh et al. 2001). Likewise, Xu et al. found that a mutation in CKIδ can also cause FASPS by modulating PER stability (Xu et al. 2005). Additionally, chemical biology approaches identified several compounds that shorten or lengthen circadian period (Chen et al. 2012; Hirota et al. 2008; Isojima et al. 2009). One remarkable example is a series of CKIδ/ε inhibitors, which can lengthen molecular clock period from 24 h to 48 h at the cellular level (Isojima et al. 2009).

How PER phosphorylation controls circadian period is still mysterious, but phosphorylation affects PER stability. PER protein is degraded by proteasome-mediated proteolysis when phosphorylation of PER triggers recruitment of βTrCP,

a subunit of the SCF ubiquitin ligase (Eide et al. 2005; Shirogane et al. 2005). However, the FASPS mutation site is different from the region involved in βTrCP recognition of PER (Eide et al. 2005). Furthermore, several results imply that phosphorylation on FASPS-mutated site stabilizes PER protein (Shanware et al. 2011; Vanselow et al. 2006; Xu et al. 2007). Therefore, phosphorylation may regulate the stability of PER in multiple ways. Recent studies of *Drosophila melanogaster* PER and *Neurospora crassa* FRQ (a functional counterpart of PER) show that multisite phosphorylation induces conformational changes in these proteins (Chiu et al. 2011; Querfurth et al. 2011). A similar case might also be true for mammalian PER: phosphorylation may control the stability of mammalian PER by changing its global structure, not just by creating a recognition site for βTrCP at a specific location.

The stability control of PER also may contribute to delay for transcriptional negative feedback. Unlike other clock genes, the expression peak of *Per1* and *Per2* mRNA is ~4 h earlier than PER1/PER2 proteins (Pace-Schott and Hobson 2002). This delay between mRNA and protein may be one of the determinants of period length.

4.2 Stability Control of CRY in Circadian Oscillations

Recently, researchers noticed that not only PER but also CRY stability is important for clock period. In 2007, two lines of ENU-mutant mice with long behavioral rhythms were reported from different groups—*Overtime* (Siepka et al. 2007) and *Afterhours* (Godinho et al. 2007). Both the *Ovt* and *Afh* mutations are located in the same gene *Fbxl3*. *Fbxl3* encodes an ubiquitin ligase E3 and controls CRY stability by inducing CRY protein ubiquitination and degradation (Godinho et al. 2007; Siepka et al. 2007). Delayed expression of CRY1 could be caused by the combinatorial effect of delayed transcription activation and active degradation. These data suggest that temporal control of clock gene products (like PER and CRY) is also important for generating circadian rhythms. Effects of the $CKI\varepsilon^{tau}$ and $Fbxl3^{Afh}$ mutations are additive and independently contribute to circadian period (Maywood et al. 2011).

4.3 Post-Translational Oscillation of the Mammalian Circadian Clock

Phosphorylation-dependent degradation may be directly related to PER oscillation. Two reports showed that PER2 protein translated from constitutively expressed mRNA undergoes circadian oscillation (Fujimoto et al. 2006; Nishii et al. 2006). These studies imply that a layer of post-translational control blankets the

transcription-translation circadian machinery. Consistent with this idea, several studies have shown that circadian rhythmicity is robust against fluctuations in oscillating transcriptional activity. For example, the expression pattern of *Bmal1* and *Clock* can be constant throughout the circadian cycle (von Gall et al. 2003). Reducing the overall transcriptional activity only modestly affects the period length of circadian rhythms in cultured cells (Dibner et al. 2009). Even in for CRY, rhythmic expression is dispensable for circadian oscillation to a certain extent; weak circadian oscillations can be observed in $Cry1^{-/-}$:$Cry2^{-/-}$cells rescued by *Cry1* under constant expression (Ukai-Tadenuma et al. 2011) or a constant supply of CRY proteins (Fan et al. 2007). Genetic studies in *Drosophila* show that flies with constant expression of PER maintain circadian rhythmicity (Ewer et al. 1988; Frisch et al. 1994; Vosshall and Young 1995; Yang and Sehgal 2001). Taken together, these results suggest that circadian oscillations do not necessarily depend solely on the transcriptional activity in the E/E′-box feedback loop, because post-translational control of clock proteins can compensate for loss of transcriptional rhythms.

A post-translational circadian oscillator was also found in the cyanobacterium circadian clock. Oscillations occur in the phosphorylation state of KaiC, a central component of cyanobacterial circadian clock, even after the termination of global transcriptional activity (Tomita et al. 2005). This KaiC-phosphorylation rhythm can be reconstituted in vitro by mixing KaiC and its regulatory factors KaiB and KaiC together with ATP (Nakajima et al. 2005). In mammals, a recent study discovered the presence of the circadian oscillations in the redox state of enucleated human red blood cells (O'Neill and Reddy 2011). The circadian oscillation in redox status of peroxiredoxin proteins is conserved from prokaryotes to eukaryotes (Edgar et al. 2012) and can regulate the neuronal activity of SCN (Wang et al. 2012). Although a core post-translational circadian oscillator in mammals remains to be identified, cooperation of transcription-translation oscillator and post-transcriptional oscillator would provide a more robust circadian timekeeping system. The investigation of compatible interactions between delayed negative feedback loops mediated by the CCEs and yet-unknown core post-translational oscillators will lead to a new understanding of mammalian circadian clocks.

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Genome-Wide Analyses of Circadian Systems

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Abstract Circadian gene expression is a pervasive feature of tissue physiology, regulating approx. 10 % of transcript and protein abundance in tissues such as the liver. Technological developments have accelerated our ability to probe circadian variation of gene expression, in particular by using microarrays. Recent advances in high-throughput sequencing have similarly led to novel insights into the regulation of genes at the DNA and chromatin levels. Furthermore, tools such as RNA interference are being used to perturb gene function at a truly systems level, allowing dissection of the clockwork in increasing depth. This chapter will highlight progress in these areas, focusing on key techniques that have helped, and will continue to help, with the investigation of circadian physiology.

Keywords Transcriptomics • Genomics • Systems biology • Clock • Circadian • ChIP-chip • ChIP-seq • RNA-seq • Interferomics • Proteomics • Metabolomics

1 Introduction

Dynamic changes in the topology of genomes and transcriptomes are not a newly recognized phenomenon—plasticity in DNA and RNA has long been recognized as a key regulatory point in most biological processes. However, on a 24-h timescale, it is only recently that the extent of changes at a systems level has begun to be appreciated (Reddy and O'Neill 2010). Progress in this arena has largely been propelled by advances in technology, which have allowed interrogation of DNA and RNA over time, at a truly global level (Akhtar et al. 2002; Panda et al. 2002; Rey et al. 2011).

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The numbers of genes and transcripts regulated in a circadian fashion are not trivial. Various studies have estimated that approx. 10–15 % of mammalian transcripts undergo circadian oscillation in tissues such as the liver or heart and similar numbers of proteins oscillate over the circadian cycle (Akhtar et al. 2002; Panda et al. 2002; Storch et al. 2002; Ueda et al. 2002). Thus, when cells and tissues are viewed at the genomic, transcriptomic, and proteomic levels, they are not only in a state of flux because of their need to maintain body homeostasis but also because of the background influence of the circadian clock on the production of these macromolecules (Hastings et al. 2003; Reddy et al. 2006). In the extreme case, as occurs in cyanobacteria, the entire genome undergoes rhythmic change, which in turn sculpts the gene expression profile of thousands of genes (Johnson et al. 2008; Vijayan et al. 2009; Woelfle et al. 2007).

In this chapter, I will discuss recent advances in our understanding of the clockwork at the genomic and transcriptomic level, highlighting the importance of considering the circadian clock in analyses of cells and tissues for pharmacological experiments. I will also touch upon the types of high-throughput approaches that have been utilized to probe the clockwork at a systems level, which will have relevance to the pharmacologically minded scientist.

2 Genomic Level Analyses of the Clockwork

The genomic landscape is traditionally regarded as static and only subject to change when cells need to undergo fundamental, and often terminal, changes such as end differentiation (Kouzarides 2007). However, this line of thought has been challenged recently by observations that genome-level changes to DNA occur in disparate organisms, from bacteria to mammals. For example, in the circadian biologist's favorite cyanobacterium, *Synechococcus elongatus*, its entire genome undergoes rhythmic supercoiling over a day, directing rhythmic abundance of mRNA (Kucho et al. 2005; Vijayan et al. 2009; Woelfle et al. 2007). In mammals, the changes are not thought to be as far reaching, but have been demonstrated at specific genomic loci where clock-relevant transcription factors, such as CLOCK and BMAL1, bind and modulate chromatin structure in a rhythmic manner (Ripperger and Schibler 2006). This has obvious implications for understanding RNA dynamics but also underscores the need for careful regard to sampling time in any experiment, since it cannot be assumed that the genome is "static" in terminally differentiated tissues or in cells cultured under laboratory conditions.

2.1 ChIPing Away at Chromatin

There are several techniques that can be applied to investigate the state of the genome at a given time, but one of the most versatile is chromatin

immunoprecipitation (ChIP). The premise of this technique is straightforward. As transcription factors (or other proteins such as histones) bind to their native targets in the genome, they are first "frozen" in time and space by using a cross-linking reagent (usually formaldehyde). The result is that all transcription factors remain attached to the DNA they were bound to prior to fixing with the formaldehyde, and any unbound protein is cross-linked to other proteins. Cross-linked chromatin thus consists of DNA with transcription factors bound to specific regions. The cross-linked can subsequently be reversed by overnight incubation at moderate temperatures (65 °C typically), and pure DNA is extracted from this subsequently (Farnham 2009).

This process can be performed on samples from different times in the circadian cycle such that a temporal map of transcription factor binding can be obtained. If a particular genomic locus is of interest, such as a promoter/enhancer region of a gene, the polymerase chain reaction (PCR) can be used to amplify the relevant target region, and its enrichment at different time points can be compared quantitatively using real-time PCR (qPCR). If, however, the target regions in the genome are unknown, or you wish to take an unbiased approach to finding transcription factor target sites, then other genome-level methods have to be used in combination with ChIP.

2.2 ChIP-chip and ChIP-seq Allow Whole-Genome Analyses of Transcription Factor Binding

Having the ability to temporally map binding sites of transcription factors (or other proteins that bind to DNA) across the genomic landscape has only recently become possible with the advent of new technologies. The first breakthrough technology was the DNA microarray. When using ChIP coupled with microarrays (also known as a DNA "chip"), the technique is termed "ChIP-chip." An important aspect is to ensure adequate genomic coverage. This is, however, difficult given the limited capacity and packing density of probe DNA sequences on the surface of microarrays and the necessity to synthesize a plethora of probes to cover the entire genome (Buck and Lieb 2004; Scacheri et al. 2006; Wu et al. 2006). To get useful coverage, multiple microarrays have to be used, which is both expensive and experimentally time-consuming. Initially, promising studies were confined to the detailed analysis of individual chromosomes, but low-resolution studies at the "whole genome" level have been performed (Bieda et al. 2006; Horak et al. 2002).

Microarrays have now been effectively usurped, as will no doubt be the case soon for transcriptomics studies, by high-throughput sequencing approaches. This technology has revolutionized genomic-scale analyses. Instead of having a defined set of probes that cover the entire genome, it is now possible to instead simply sequence all of the ChIPed DNA sequences that represent binding regions across the genome (termed "ChIP-seq"). After some relatively demanding bioinformatics

to align each sequence to the reference genome (e.g., mouse or human), each sequence, and the relative number of sequences at a particular genomic locus, can be determined at a very high resolution (Farnham 2009). At binding sites, enrichment of overlapping sequences yields "peaks" where the target protein was bound (Fig. 1). Further bioinformatics can subsequently determine, with ever-increasing precision, binding motifs within the genomic DNA (Park 2009; Pepke et al. 2009).

Recently, some investigators have applied ChIP-seq methodology to investigate the function of the core transcription–translation feedback oscillator at a genomic scale. For example, by using an antiserum directed against BMAL1, Rey and colleagues have mapped BMAL1 binding sites across the genome of mouse liver, over the circadian cycle (Rey et al. 2011). This convincingly showed that BMAL1 rhythmically binds to over 2,000 target sites in the genome, with peak occupancy occurring in the middle of the circadian day. Functionally, these targets were diverse, but their results pointed towards carbohydrate and lipid metabolism loci as major targets for BMAL1's action in vivo. Furthermore, using a combination of bioinformatics and modeling, these authors were able to show that E-box motifs were strongly correlated with the presence of BMAL1 binding sites and to rhythmic transcription of these loci (Rey et al. 2011). Recent comprehensive studies have further developed these principles and determined a circadian "chromatin landscape" by assaying other components of the "clock complex" (Koike et al. 2012). The true power, however, of genome-level approaches comes from the ability to relate changes at the level of DNA to transcripts and eventually to proteins, the effectors of cellular physiology.

3 Circadian Transcriptomics

Early studies in mammals implicated a "core" set of clock genes in the molecular clockwork (Buhr and Takahashi 2013; Hastings et al. 2003; Reddy and O'Neill 2009). Amongst these were the *Period* and *Cryptochrome* gene families, as well as the transcription factors driving their expression, *Clock* and *Bmal1*. It became apparent quickly that the majority of these genes were expressed rhythmically and that there were close parallels between the mammalian clockwork and what had been extensively investigated in *Drosophila* previously (Hastings et al. 2003). Initially, however, it was thought that relatively few genes (and their respective transcripts) were under circadian clock control. However, with the advent of microarray technology, this hypothesis became eminently testable.

The first study to map the circadian transcriptome was performed in plants by Harmer and colleagues (Harmer et al. 2000). This demonstrated the pervasive nature of rhythmic transcription and the clear anticipatory advantage that clock control over plant homeostasis might have. It did not take long for similar results to emerge in other eukaryotic systems, most notably in mammals.

Several studies illustrated the extensive influence of the circadian clock on tissue transcriptomes, most notably in the liver, brain, and heart (Akhtar et al. 2002; Panda

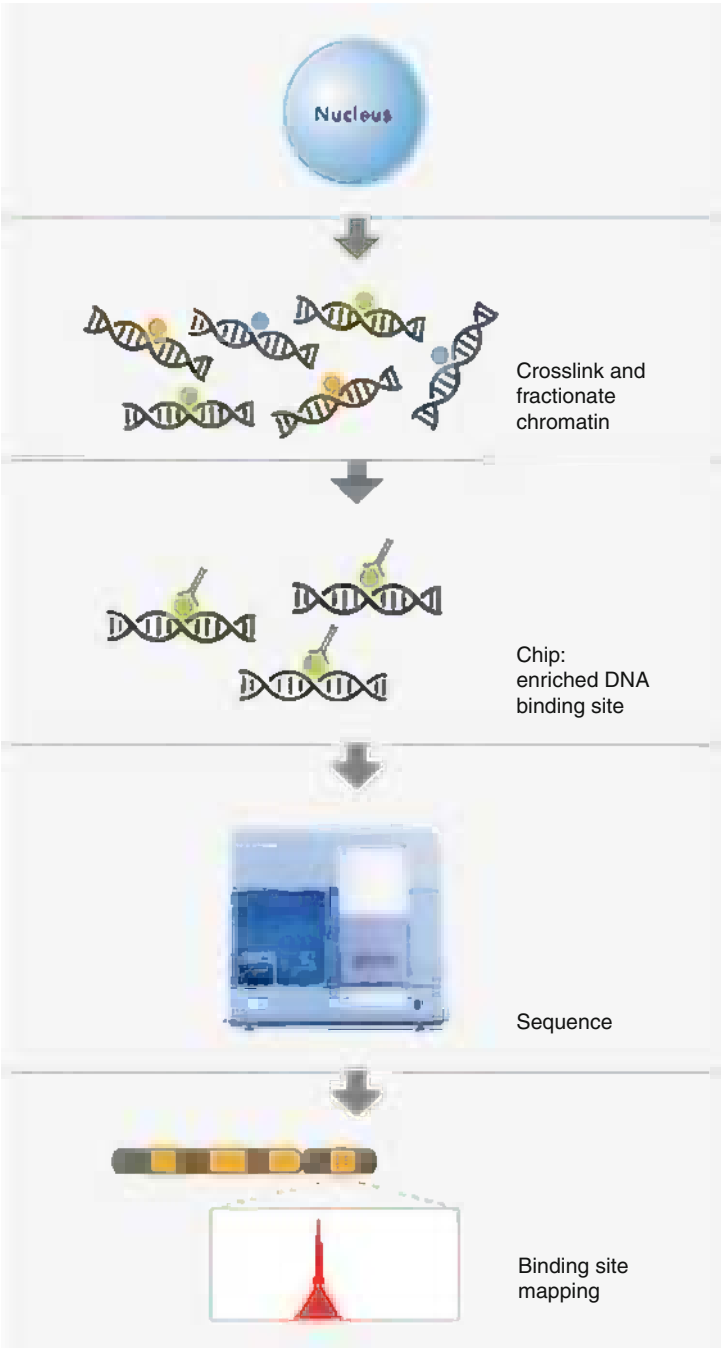

Fig. 1 Protein–chromatin interactions are first cross-linked in situ using, typically, formaldehyde. Specific DNA fragments are co-immunoprecipitated and sequenced to identify genome-wide sites associated with a factor or modification of interest (Adapted from Illumina Web site, http://www.illumina.com/technology/chip_seq_assay.ilmn)

et al. 2002; Storch et al. 2002; Ueda et al. 2002). Subsequently, a plethora of tissue transcriptomes has been mapped over the circadian cycle, including those of adipose tissue, gut, and bone (Polidarova et al. 2011; Zvonic et al. 2006, 2007). Together, these studies and others highlight that in excess of 10 % of the transcriptome in any individual tissue could undergo robust rhythmic change over the circadian day and night (Hughes et al. 2009). The functional consequences of this widespread control over gene expression by the clock are perhaps obvious but are only beginning to be recognized more widely outside the circadian clock field (Fig. 2).

As microarray technology has matured, high-throughput sequencing seems set to take over its reign over transcriptomics research, in a similar way in which ChIP-chip has given way to ChIP-seq. The power of sequencing the transcriptome (after reverse transcription and processing into DNA) is clear (Hawkins et al. 2010; Marguerat and Bahler 2010; Wang et al. 2009). RNA-seq (the term used to describe this technique) is so powerful because it cannot only interrogate messenger RNA (mRNA) but can also be used to assay small RNAs, such as microRNAs (Cheng et al. 2007; Gatfield et al. 2009; Yang et al. 2008) and other noncoding RNA species (e.g., large noncoding RNA—lncRNA). Furthermore, RNA-seq can be used to perform systems-level analyses of alternative splicing, which may add further tiers of regulation to RNA processing by the clock (Licatalosi et al. 2008; Wang et al. 2010).

4 Interferomics and Manipulating the Clockwork

Interferomics is a novel area within systems biology that aims to study the biological impact of perturbing post-transcriptional (but pre-translational) processes (Baggs and Hogenesch 2010). The main tool that is becoming used increasingly in this area is RNA interference (RNAi). Using this technique, it is possible to silence a specific gene with a cell line using a small interfering RNA molecule (siRNA). With a suitable screening platform for circadian clock function, a collection of these siRNAs could be applied onto cells and phenotypes screened for using a suitable screening assay.

The pre-eminent assay system used by circadian biologists to assay the clockwork employs bioluminescent reporter constructs (Yamaguchi et al. 2001). These consist of “clock gene” promoters driving the expression of luciferase expression (e.g., *Bmal1*::*luciferase* and *mPer2*::*luciferase*) which act as markers for circadian oscillation within the cell line. Once reporters are introduced into cells stably, siRNAs can be transfected into reporter cells and their effects on the clock determined using real-time bioluminescence monitoring (Hastings et al. 2005).

This type of approach has recently been put into practice in two major studies (Maier et al. 2009; Zhang et al. 2009), with similar paradigms also used for chemical compound screening (Hirota et al. 2010). Interestingly both kinds of approach have delineated links to canonical kinase pathways, including casein

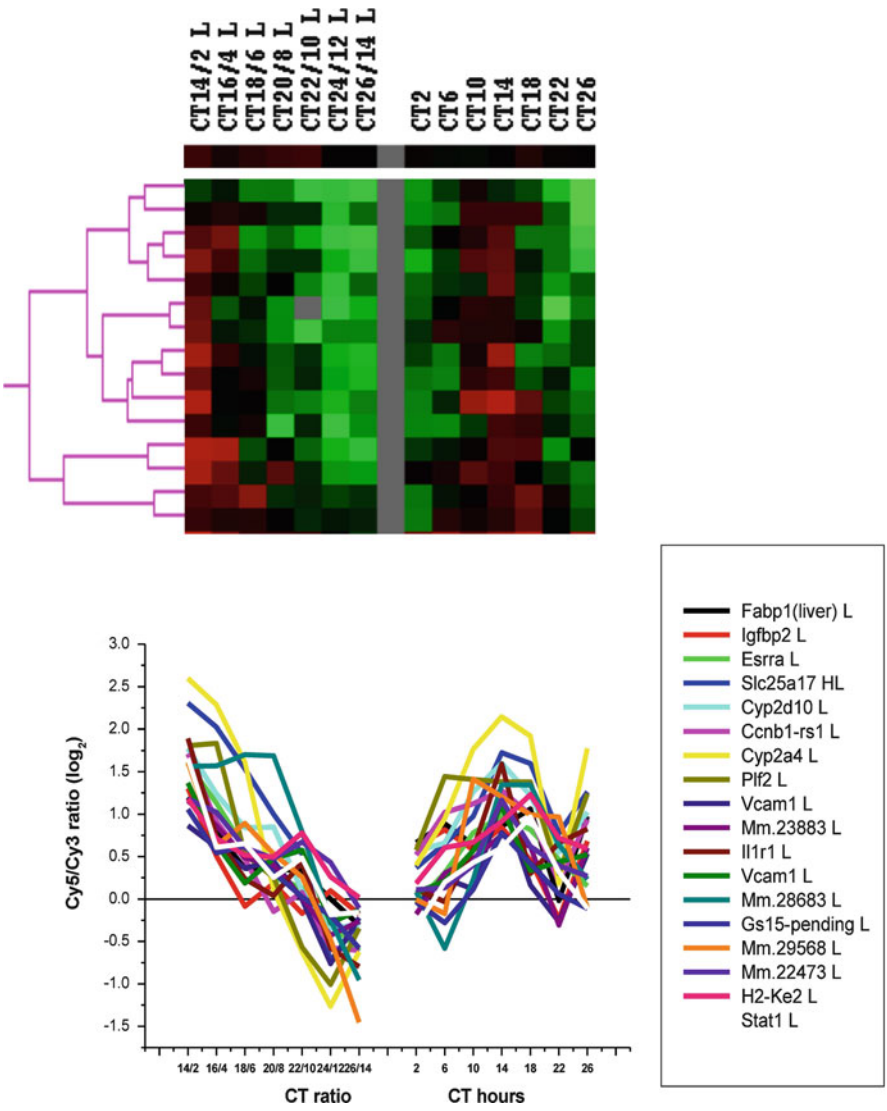

Fig. 2 Microarray analysis of gene expression over a circadian cycle (i.e., in the absence of external time cues) in mouse liver. The *top panel* shows a heat map with genes that oscillate in a similar pattern clustered together. In this case, transcripts peaking in the middle of the cycle, CT12, are shown (*CT* circadian time; where CT0 is subjective dawn and CT12 is subjective dusk). The *bottom panel* shows the same data as a graphical representation for each gene. The *left side* of the heat map and graph assayed expression by an autocorrelation method. See Akhtar et al. (2002) for further details

kinases (Hirota et al. 2010; Maier et al. 2009). This highlights the power of complementary approaches to dissecting components of the transcription–translation feedback loop.

5 Beyond Transcription

Proteins are of course the final effectors of cellular function; what do we know about the impact of rhythmic transcription on protein levels following translation? Intuitively, this would seem to be quite a straightforward question. However, the data in the clock field and in other domains highlights that transcriptomics datasets do not correlate well with proteomics datasets from the same samples (Hanash 2003; Reddy et al. 2006), emphasizing the importance of mapping protein abundance in addition to mRNA expression. This point is further highlighted by recent data from Selbach and colleagues, who took a systems approach to determine the "flow" of mRNA to protein quantitatively (Schwanhausser et al. 2011). More detailed descriptions of post-translational aspects (e.g., proteomics and metabolomics) are considered in other chapters within this volume (Robles and Mann 2013).

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Proteomic Approaches in Circadian Biology

Maria S. Robles and Matthias Mann

Abstract Circadian clocks are endogenous oscillators that drive the rhythmic expression of a broad array of genes that orchestrate metabolism and physiology. Recent evidence indicates that posttranscriptional and posttranslational mechanisms play essential roles in modulating circadian gene expression, particularly for the molecular mechanism of the clock. In contrast to genetic technologies that have long been used to study circadian biology, proteomic approaches have so far been limited and, if applied at all, have used two-dimensional gel electrophoresis (2-DE). Here, we review the proteomics approaches applied to date in the circadian field, and we also discuss the exciting potential of using cutting-edge proteomics technology in circadian biology. Large-scale, quantitative protein abundance measurements will help to understand to what extent the circadian clock drives system wide rhythms of protein abundance downstream of transcription regulation.

Keywords Circadian rhythm • Proteomics • Mass spectrometry • Protein quantification • Posttranslation modifications

Abbreviations

CE-MS	Capillarity electrophoresis mass spectrometry
GC-MS	Gas chromatography mass spectrometry
LC-FT MS/MS	Liquid chromatography Fourier transformation tandem mass spectrometry
MALDI TOF MS	Matrix-assisted laser desorption/ionization time of flying mass spectrometry

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MS	Mass spectrometry
SELDI	Surface-enhanced laser desorption/ionization
SPE	Solid-phase extraction

1 Introduction

Genetic technologies have long been used to study circadian biology, as reviewed in the previous chapter (Reddy 2013). In contrast, proteomic approaches have so far been limited and if applied at all, have used two-dimensional gel electrophoresis (2-DE). Due to its technical limitations and to recent advances in high-resolution mass spectrometry (MS), this technology is becoming obsolete. Currently, the most powerful proteomics method is high-accuracy, quantitative MS shotgun proteomics. In that approach, the proteome is digested into peptides, and the resulting, very complex mixtures are separated by liquid chromatography (LC), which is coupled to high-resolution tandem mass spectrometric identification and quantification (MS/MS). MS-based proteomics is increasingly used to determine global protein abundance, protein–protein interactions, as well as posttranslational modifications (PTMs) (Aebersold and Mann 2003; Cox and Mann 2011; Mallick and Kuster 2010; Yates et al. 2005).

Here we review the proteomics approaches applied to date in the circadian field; these can be classified into three general methods: expression proteomics, interaction proteomics, and proteomics of PTMs. Moreover, we also discuss the exciting prospect and potential of using cutting-edge proteomics technology in circadian biology.

2 Expression Proteomics

Expression proteomics is defined as the measurement of the absolute or relative quantity of the proteins in a sample. Therefore, the notion of expression proteomics is similar to the widely applied transcriptomics approaches such as microarray measurements or, more recently, "deep sequencing." However, the goal of proteomics is to ideally measure the total protein complement of a biological system. In principle, this has the advantage over transcriptomics that the proteins are generally the functional units in the cell rather than intermediates.

MS-based expression proteomics relies on quantifying the mass spectrometric signals of the peptides to determine protein abundance in a complex mixture. The two most common methods are isotope labeling and label-free quantification (Bantscheff et al. 2007). The isotope-based methods make use of the fact that MS

can readily quantify the ratios between versions of the same peptide that have different mass. The stable isotopes can be introduced by chemical methods or by metabolic labeling. Among the latter method, stable isotope labeling by amino acids in cell culture (SILAC) is the most accurate and widely used; it relies on the incorporation of a "heavy" or "light" nonradioactive isotope of an essential amino acid into all proteins of the proteomes to be compared (Ong et al. 2002; Ong and Mann 2006). These proteomes are mixed, processed, and analyzed together. The heavy SILAC-labeled proteome can easily be distinguished from the light-labeled proteome by modern high-resolution MS techniques on the basis of the known peptide mass differences. The relative intensity between the peaks of the SILAC peptide pairs directly reflects the relative abundance of the protein in the original samples. This method can be very accurate because it eliminates quantitative differences due to sample processing. Although SILAC was originally developed for cell culture experiments, it has recently been applied to whole organisms and human tissues (Baker et al. 2009; Geiger et al. 2010; Kruger et al. 2008). Alternatively, protein quantification can also be achieved with label-free methods based on alignment of separate LC-MS/MS runs of peptide mixtures and comparison of the signal intensities of the same peptides between the runs. Label-free quantification is less accurate than isotope labeling, especially if several fractionation steps are involved; however, it is simpler and can be applied to any system.

For many years circadian regulation of metabolism and physiology has been investigated through the analysis of gene expression. This has been made possible by DNA array technology, which has facilitated large-scale circadian gene expression studies and provided essential information about circadian transcriptional control in mouse brain and peripheral tissues (Duffield 2003; Hughes et al. 2009; McCarthy et al. 2007; Panda et al. 2002; Storch et al. 2002).

By comparison, global proteomic analyses in the circadian field have been very limited in number and in the depth of coverage (Table 1). The majority of these analyses employed comparative two-dimensional gel electrophoresis (2-DE) and, specifically, difference gel electrophoresis (2-DIGE). Below we review proteomic reports characterizing daily variation of protein levels in several rodent organs mainly using that technology, which is now out of date and not recommended anymore. We also discuss the identification of circadian and electrical stimulation-dependent released peptides in the suprachiasmatic nucleus (SCN).

2.1 Proteomes of Brain and Eye

The circadian timekeeping system in mammals is organized in a hierarchical manner (Buhr and Takahashi 2013). Virtually, all tissues contain internal, self-autonomous clocks that regulate local physiology and metabolism (Stratmann and Schibler 2006). A master clock located in the brain, the suprachiasmatic nucleus (SCN), synchronizes and phase-dependently coordinates the peripheral clocks (Ko and Takahashi 2006). The SCN in turn receives light cues from the retina

Table 1 Summary of expression proteomics approaches applied to circadian biology

Tissue	Technique	Identified/rhythmic proteins	References
SCN	2-DIGE/MS	871/34	Deery et al. (2009)
SCN light stimulated	LC MS/MS	2,131/387	Tian et al. (2011)
SCN releasate RTH-stimulated	SPE-MALDI TOF MS	14	Hatcher et al. (2008)
SCN endogenous peptides	LC-FT MS	102	Lee et al. (2010)
Retina	LC-MALDI TOF MS LC-MS/MS	415/11	Tsuji et al. (2007)
Rat pineal gland	2-DE MALDI TOF	1,747/60	Moller et al. (2007)
Liver	2-DIGE MALDI TOF MS LC-MS/MS	642/39	Reddy et al. (2006)
Blood	SELDI MS LC-MS/MS	6	Martino et al. (2007)

through the retinohypothalamic tract (RHT), entraining it in phase to the external environment. This information is then transmitted by the SCN via humoral and neuronal signals to peripheral tissues, thus synchronizing behavior and physiology in the whole organism (Davidson et al. 2003). Expression profiling by microarrays in the SCN has identified hundreds of transcript that appear to be under circadian control (Panda et al. 2002; Ueda et al. 2002; for a review see Reddy 2013). Only recently has the SCN been the target of proteomic analyses to uncover rhythmic oscillations of intracellular as well as extracellular, secreted molecules.

Hastings and colleagues performed 2D-DIGE analyses with protein extracts from mouse SCN collected every 4 h across the circadian day (Deery et al. 2009). On the resulting gels, 871 protein spots were detected; among them, 115 showed circadian variation. Of these, 53 spots were analyzed using protein digestion followed by liquid chromatography and tandem mass spectrometry (LC-MS/MS). Since some of the analyzed spots corresponded to isoforms of the same protein, only 34 unique proteins showing robust circadian changes were finally identified. The authors estimated that between 6 % and 13 % of SCN soluble proteins show daily oscillation, a proportion notably higher than the previously reported SCN cycling transcriptome (5 %) (Ueda et al. 2002). Moreover, only 11–38 % of the cycling proteins also showed rhythmicity at the transcript level, which was interpreted to imply a key role of posttranscriptional regulation in the circadian clock (Deery et al. 2009). Oscillating SCN soluble proteins covered diverse functional categories with considerable overrepresentation of molecules involved in synaptic vesicle recycling. Further experiments described in the study demonstrated the importance of vesicle recycling factors in the maintenance of electrical rhythmicity as well as neuronal circuitry, both known and central properties of the SCN (Weaver 1998).

This analysis complemented previous work describing MS-characterized rat SCN releasates across circadian time and after electrophysiological stimulation of the RHT (Hatcher et al. 2008). Secreted neuropeptides collected and concentrated on solid-phase extraction (SPE) materials were analyzed by off-line MALDI TOF MS. SCN releasates were found to contain several established circadian neuropeptides as well as some peptides that could not be identified because their masses did not match known compounds. Intriguingly, this work revealed that the releasate content is stimulation specific, characterized by a robust secretion of proSAAS-derived peptides after RHT stimulation. The role of one of these peptides in light-mediated cues was demonstrated by inducing an SCN phase shift response after exogenous application. A more recent study applied liquid chromatography in combination with high-resolution MS (LC-FTMS/MS) to detect neuropeptides in the rat SCN (Lee et al. 2010). The authors identified 102 SCN endogenous peptides in addition to 12 peptides bearing different posttranslation modifications (PTMs).

It is known that photic input induces transcriptional activation in the SCN with de novo transcription of immediate early and clock genes as well as other light-induced genes that ultimately mediate the phase reset of the clock (Albrecht et al. 2001; Araki et al. 2006; Castel et al. 1997; Porterfield et al. 2007). To investigate the effects of light stimulation on proteome-wide expression in the mouse SCN, Figeys and coworkers recently developed a much more sophisticated proteomic approach termed AutoProteome (Tian et al. 2011). It consisted of an automatic sample processing step followed by LC-MS/MS with two stages of peptide separation. This study demonstrated for the first time that light stimulation induces significant changes in the SCN proteome, as 387 proteins of a total of 2,131 quantified ones showed light-induced changes in their expression levels. Bioinformatic analysis indicated that light-inducible proteins are widely distributed into diverse canonical pathways. Among the light-responsive proteome, the authors selected several for confirmation. Two of these proteins were already previously associated with clock timing processes, vasopressin-neurophysin 2-copeptin, and casein kinase 1 delta, and three of them (Ras-specific guanine nucleotide-releasing factor, the deubiquitinating enzyme USP9X, and the ubiquitin-protein ligase UBE3A) had no previously recognized connection to the circadian clock. Moreover, the analysis showed enrichment of proteins from the ubiquitin and proteasome pathways indicating their potential role in controlling protein expression in the SCN connected to the light-resetting response.

Before reaching the SCN, light information is received and processed in the retina by photoreceptors and retinal ganglion cells. Besides being the essential organ for photic entrainment in mammals, the retina was the first peripheral organ where an intrinsic clock was identified (Tosini and Menaker 1996). Gene expression studies have indicated that circadian rhythms in the retina regulate many aspect of its physiology (Kamphuis et al. 2005; Storch et al. 2007). Furthermore, a proteomic analysis of the retina using 2-DE combined with MALDI TOF MS and LC-MS/MS identified 11 proteins with circadian oscillations (Tsuji et al. 2007). Despite the limited number of identification, rhythmic proteins covered different

biological functions suggesting that a broad range of physiological processes may be controlled at the protein level in the retina by the circadian clock.

Characterization of protein oscillations in the rat pineal gland has been the focus of another proteomic report (Moller et al. 2007). In mammals the pineal gland controls the circadian synthesis and secretion of the hormone melatonin. Production of melatonin peaks at night and its elevated nocturnal plasma level is used as an indicator of the photoperiodic time (Goldman 2001). Employing 2-DE followed by MALDI TOF MS/MS identification, the study identified 60 proteins with differential expression between day and night in the rat pineal gland (Moller et al. 2007). A total of 25 proteins were found to be up-regulated at night, which is the peak of the synthesis of the hormone. Bioinformatic classification showed that proteins highly expressed at night are involved in morphogenesis and local metabolism. Additionally and previously unreported, several proteins showed high expression during the day, suggesting a distinct rhythmic metabolism in anti-phase to the melatonin production.

2.2 Proteomes of Peripheral Organs

Proteomics has also been applied to understand circadian regulation of local metabolism in peripheral organs. Gene expression profiling has long illustrated the role of circadian transcription in the control of physiology in different mammalian peripheral organs. Early reports have identified hundreds (Akhtar et al. 2002; Panda et al. 2002; Storch et al. 2002; Ueda et al. 2002) and a more recent one thousands (Hughes et al. 2009) of rhythmic transcripts in the mouse liver (Reddy 2013; Brown and Azzi 2013). To complement gene expression analysis, Reddy et al. performed the first circadian proteomic study to identify protein oscillations in the mouse liver (Reddy et al. 2006). Proteins collected every 4 h across the circadian cycle were analyzed by 2D-DIGE and identified by MALDI TOF-MS or LC-MS/MS. The authors detected 642 proteins, 60 of which oscillated with high statistical significance, whereas 20 % of the identified proteins showed overall significant daily changes, a rate that differed notably from the reported circadian transcriptome (5–10 %) (Akhtar et al. 2002; Hughes et al. 2009; Panda et al. 2002; Storch et al. 2002; Ueda et al. 2002). Additional validation revealed that almost half of the proteins found to be rhythmic did not have a cycling transcript, which would be consistent with a key role of posttranscriptional regulation in the circadian clock. Similar divergences between transcriptome and proteome have been previously reported in cancer (Hanash 2003) and many other systems, but more recent studies using high-precision instrumentation have generally shown higher mRNA–protein correlation (Cox and Mann 2011).

Interestingly, different protein isoforms encoded by individual genes were found among rhythmic liver proteins in the study. In addition, rate-limiting enzymes involved in central liver metabolic pathways like carbohydrate metabolism and the urea cycle showed daily oscillations at the protein level. This highlights the fundamental role of circadian regulation of protein expression in liver physiology. Further knowledge of hepatic metabolism regulation by circadian clocks at the level of proteins could contribute greatly to the understanding of pathologies of this organ. Furthermore, it would aid in the development of chronotherapeutic strategies aimed at minimizing drug toxicity (Akhtar et al. 2002; Sewlall et al. 2010).

SELDI and LC-MS/MS were used in an attempt to detect daily changes in peptides in mouse blood (Martino et al. 2007). This study attempted to find markers that could define body time of day, as indicative of changes in the organism's metabolism. While few peptides were identified, the potential of following daily changes in blood protein abundance in humans to monitor health and diseases is indisputable. In the future, using more modern proteomics technology, it may be possible to find such markers, which could then be applied in molecular diagnosis of aberrant clock function or to chronotherapy applications.

2.3 Current Capabilities of Expression Proteomics

Until now, daily dynamic expression of proteins has been mostly investigated with 2-DE followed by MS. This technique has several shortcomings, first of all limited resolution and throughput, leading to quantification of only a small subset of the proteome. Specifically, only soluble proteins can be assayed; analyzed proteins have to be detected as spots in the gels; and finally, these spots need to be identified one by one using MS. In addition to these general restrictions, 2-DE faces additional challenges when characterizing temporal changes of protein abundance: the spots need to be detected in all or the majority of the assayed gels, spot localization needs to match between gels (but PTMs alter the electrophoresis mobility of the proteins), and relative changes in their intensity have to be estimated by gel image analysis. In contrast, high-resolution MS-based quantitative proteomics enables drastically deeper proteome coverage as well as much more accurate comparison of protein abundance between samples. For example, feasibility of high-resolution, quantitative MS-based proteome analysis has been demonstrated by the quantification of more than 5,000 proteins in embryonic stem cells (Graumann et al. 2008). This method can also quantify tissue proteomes to considerable depths, by using either the SILAC mouse (Kruger et al. 2008) or SILAC "spike-in" strategies (Geiger et al. 2011). Proteome coverage of tissues is somewhat reduced compared to cell lines, mainly due to the large differences in protein abundance in the sample (dynamic range). Nevertheless, measurement of global changes in more than 4,000 proteins between young and old mice has already been

described (Walther and Mann 2011). These numbers still compare favorably to results from early gene arrays, and it is clear that this technology could be uniquely useful for comparative analysis of circadian transcriptome and proteome.

3 Interaction Proteomics

Novel mammalian clock components and modifiers have often been discovered by genetic screens (Takahashi et al. 2008; Buhr and Takahashi 2013). In particular, essential clock proteins were identified by using forward and reverse genetics, and more recently, chemical and functional genomics have revealed novel regulators; see review by Baggs and Hogenesch (2010). Proteomics can, in principle, complement these approaches through the determination of physical interactions of proteins involved in circadian function. However, so far there are only few examples of finding novel clock components in this way. "Interaction proteomics" is an entire field dedicated to mapping protein–protein interactions and protein complexes. The method involves affinity purification of a "bait" protein followed by MS-dependent identification of interaction partners. The lack of good quality, specific antibodies against many proteins often requires the use of tagged versions of the bait. A key concept in interaction proteomics is the necessity of quantification to distinguish specific from background interactions, reviewed in (Gingras et al. 2007; ten Have et al. 2011; Vermeulen et al. 2008). This is becoming increasingly important because high-sensitivity, high-resolution MS can identify hundreds of proteins in single pulldowns, almost all of them being nonspecific binders. Conversely, the ability to filter out unspecific binders allows mild elution conditions that retain weak and transient interactions. This in turn enables following time-dependent interactions. SILAC, chemical labeling, or label-free algorithms can all quantify the proteins (Gingras et al. 2007; Hubner et al. 2010; Sardiu and Washburn 2011; Wepf et al. 2009). In all cases the procedure requires parallel bait and control purifications (from tagged cell lines or specific antibody beads and untagged cell lines or control beads, respectively), because of the possibility of dynamic exchange of complex components in the combined samples. Peptides from unspecific binders will have similar intensities in MS spectra from both pulldowns (one-to-one ratios), while specific protein binders will be enriched relative to the control precipitation; see Fig. 1. This very powerful technology remains to be exploited for mammalian circadian biology; the examples below generally did not use MS-based quantification and instead filtered out probable unspecific binders and contaminants by comparison to control gels.

Some years ago Schibler and colleagues described the first interaction proteomics experiment using rat cell lines stably expressing exogenous tagged PER1 protein (Brown et al. 2005). Immunoprecipitation of tagged PER1 complexes from nuclear

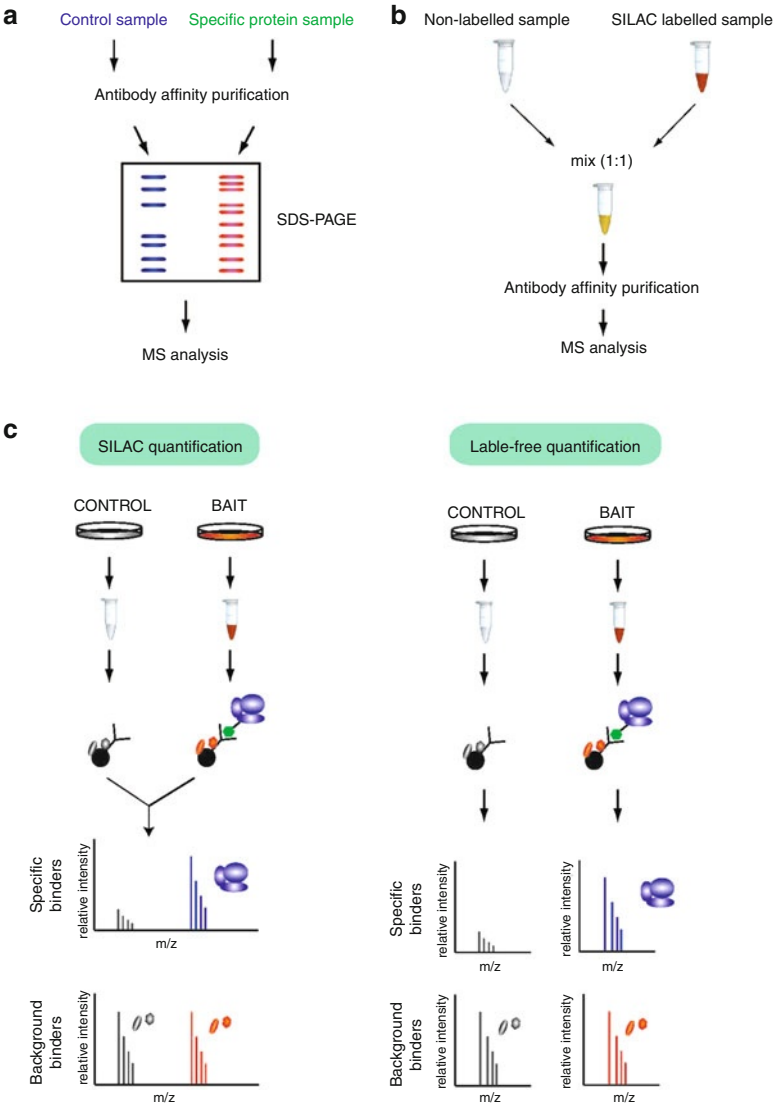

Fig. 1 Schematic representations of the interaction proteomics approaches employed in circadian biology and generally used quantitative affinity purification methods. (**a**) Workflow of the studies performed in mammals with control purification as a reference for unspecific protein pulldowns (Brown et al. 2005; Duong et al. 2011; Robles et al. 2010). (**b**) The method applied to *Neurospora*; reference sample for quantification was obtained with SILAC-labeled protein extracts (Baker et al. 2009). (**c**) Workflows of the two approaches used for quantitative interaction proteomics. *Left panel* shows the strategy followed for SILAC quantification: two cell populations, labeled with light or heavy SILAC and expressing control or bait protein, respectively, are lysed followed by affinity purification. After purification extracts are mixed and analyzed by LC-MS/MS, both light and heavy peptides will appear in the MS spectra, allowing direct comparison of intensities and therefore precise quantification. Strategy for label-free quantification is shown in the right panel.

cell extracts followed by MS analysis identified two novel interactors: NONO and WDR5. Knock-down validation studies showed that the RNA-binding protein NONO is essential for circadian rhythmicity in mammalian cells and in flies. In addition, WDR5, a member of the histone methyltransferase complex, associated to PER complexes and seemed to assist in their function (Brown et al. 2005).

More recently, two studies from the Weitz group described a more refined interaction proteomics approach in which the tagged proteins were endogenously expressed—substituting the untagged endogenous forms while preserving their function. This method ensures that the affinity-purified protein complex is the only complex present in the cell and that it is functional. In this way, novel clock regulators important for circadian function were discovered in protein complexes isolated from mouse cells and tissues (Duong et al. 2011; Robles et al. 2010). Affinity-purified nuclear protein complexes from mouse fibroblast containing tagged BMAL1 were analyzed by MS to identify RACK1 (receptor for activated C kinase 1) as a new BMAL1 interaction partner. Validation experiments showed that RACK1 binds to BMAL1 in a time-dependent manner, recruiting PKCα to the complex, and that it inhibits BMAL1-CLOCK activity. This effect could be mediated by PKCα-dependent phosphorylation of BMAL1, which was demonstrated in an in vitro assay (Robles et al. 2010). More recently, a similar interaction proteomics approach in mouse tissues identified novel components of the endogenous PER protein complexes. Importantly, this uncovered the first molecular mechanism for negative feedback in the mammalian circadian clock (Duong et al. 2011). Briefly, MS analysis identified the RNA-binding protein PSF (polypirimidine tract-binding protein-associated splicing factor) as a novel component of the PER nuclear protein complexes. PSF binds to the PER complex and functions as a transcription corepressor by recruiting SIN3A-HDAC. Consequently, PERs mediate the binding of PSF-SIN3A-HDAC in a time-dependent manner to the *per1* promoter inducing histone deacetylation and therefore transcriptional repression.

Another elegant application of proteomics was directed at the *Neurospora* circadian clock. For the first time in circadian biology, Dunlap and coworkers used SILAC-based interaction proteomics (Baker et al. 2009). To determine the dynamic interactome of the *Neurospora* clock gene FREQUENCY (FRQ), heavy SILAC-labeled *Neurospora* was used as a reference sample to assess relative protein abundance among the experimental time points. The heavy control consisted of a pooled mixture of protein lysates from six cultures collected every 4 h so as to contain all time-dependent FRQ isoforms and complexes. This pool was

Fig. 1 (continued) In this case control and bait expressing cell lysates are immunoprecipitated and analyzed by MS separately. Peptides from specific binders have different intensity between control and specific pulldown, while background proteins have similar intensities in both immunoprecipitations

mixed 1:1 to light protein lysates collected in the same time-dependent manner. The heavy-to-light (H/L) ratio of any given peptide identified by MS therefore represented the relative change in their abundance. In this way the authors defined the temporal interactome of FRQ. For instance, the FRQ-interacting RNA helicase (FRH) interacts with FRQ throughout the day, the heterodimeric transcription factor WHITE COLLAR-1 and WHITE COLLAR-2 complex (WCC) preferentially binds FRQ in the early part of the day.

4 Posttranslational Modifications in Circadian Biology

Daily rhythms are generated by a molecular mechanism consisting of negative and positive transcriptional feedback loops. Proper function of this molecular clock is regulated at multiple levels, transcription, posttranscription, translation, and posttranslation. In recent years, multiple studies have highlighted the role of PTMs in core clock components for general clock function as well as for fine-tuning (Mehra et al. 2009). An increasing number of PTMs have been described in clock proteins in different species, and furthermore, recent data in cyanobacteria indicate a clock based entirely on PTMs (Johnson et al. 2008). One of the most common PTMs of clock proteins is phosphorylation, regulation of which is temporal and phase-specific (Vanselow et al. 2006). In addition, acetylation, ubiquitination, and SUMOylation have been reported to regulate clock protein function or stability in mammals (Asher et al. 2008; Cardone et al. 2005; Lee et al. 2008; Mehra et al. 2009; Nakahata et al. 2008; Sahar et al. 2010). A common characteristic of most of the reported PTMs is their rhythmicity; for that reason measurement of PTM temporal changes would be extremely desirable in the circadian field. To date there is only one such study and it describes circadian dynamic changes of phosphorylation of the FRQ protein in *Neurospora crassa*. The data was obtained in the paper that characterized dynamic interactions of FRQ already mentioned above Baker et al. 2009. Using SILAC in combination with high-resolution MS, the authors identified and quantified 75 phosphorylation sites in the affinity-purified protein. Quantification of these sites across different circadian times allowed depicting phase-specific phosphorylation changes and demonstrated how this temporal regulation affects FRQ stability. Thus, this study showed for the first time the quantitative extent of rhythmic phosphorylation, similarly to what has been qualitatively reported for other clock proteins in different species (Chiu et al. 2008; Kivimae et al. 2008).

Technological advances in MS now allow to characterize modified peptides with high quantitative accuracy and to localize the PTM with single amino acid resolution in the peptide. Since one of the main challenges of PTM analysis is the low abundance of many modified peptides, enrichment strategies have been developed (Bantscheff et al. 2007; Choudhary and Mann 2010). Modification-specific enrichments can be done at the protein or peptide level and for the entire proteome or for specific, purified proteins of interest. In many biological fields and particularly in circadian biology, it is not only interesting to identify PTMs but even more

to determine their changes among different states of the proteome. This can be achieved by MS-based quantitative PTM analysis. Several studies have recently reported extensive dataset of different PTMs: phosphorylation (Beausoleil et al. 2004; Bodenmiller et al. 2007; Dephoure et al. 2008; Ficarro et al. 2002; Olsen et al. 2010), acetylation (Choudhary et al. 2009; Kim et al. 2006), N-glycosylation (Kaji et al. 2007; Zielinska et al. 2010), methylation (Ong et al. 2004), ubiquitination (Argenzio et al. 2011; Kim et al. 2011; Wagner et al. 2011), and SUMOylation (Andersen et al. 2009; Tatham et al. 2011). The first study of global dynamic changes in phosphorylation upon stimulation in cell lines reported more than 6,000 phosphorylation sites (Olsen et al. 2006), and a recent study quantified in vivo changes in more than 10,000 phosphosites in mouse liver upon insulin injection (Monetti et al. 2011). Additionally, this technology has been applied to the study of proteome and phosphoproteome dynamics during different stages of cell division, a system that experimentally resembles the circadian one. Remarkably, the study revealed that most of the detected phosphosites and approximately 20 % of the quantified proteins show changes during the cell cycle (Olsen et al. 2010). Additionally, phosphorylation site occupancy (or "stoichiometry") was determined for thousands of the detected phosphosites during different cell cycle stages. Estimation of phosphorylation site stoichiometry will be highly desirable also in circadian biology. This would be very interesting in particular for clock proteins, given that their progressive phosphorylation is a feature of timekeeping and that this phosphorylation kinetics determines proper clock function. Furthermore, phosphorylation deregulation in some clock proteins has been associated with disorders in humans (Reischl and Kramer 2011; Vanselow and Kramer 2007). Because several kinases play an essential role in the function of the clock (Lee et al. 2011; Reischl and Kramer 2011), determination of phosphorylation occupancy more generally in the whole circadian phosphoproteome could result in significant insights into the role of additional kinases in circadian rhythmicity.

5 Mass Spectrometry Applied to Metabolomic Studies

In addition to proteins, mass spectrometry can also be applied to the study of metabolites. Metabolomics technology, in particular gas chromatography–mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and capillarity electrophoresis-mass spectrometry (CE-MS), has been used to the study of circadian metabolomics. Daily oscillations in mouse blood and plasma as well as human plasma and urine have been reported in recent studies (Dallmann et al. 2012; Eckel-Mahan et al. 2012; Minami et al. 2009). A recent paper provides an overview of integrating other circadian datasets with metabolomics (Patel et al. 2012).

6 Perspectives for Proteomics in Circadian Biology

Proteomics is essential in the functional annotation of the genome and future attempts to build a quantitative, "systems-based" description of cell biology in general (Cox and Mann 2011) and of circadian biology in particular (Baggs and Hogenesch 2010). Circadian biology is an ideal field for the application of quantitative proteomics since circadian clocks control daily oscillations of gene expression and thereby protein abundance, modification, activity, and localization in the cell.

Circadian regulation of gene expression at the level of the transcriptome has been the focus of many studies in the circadian field. In contrast, very little is known about the circadian regulation of global protein abundance and PTMs, mainly because of the technical challenges described above. Given that proteins and not nucleic acids are the main executors of cellular functions, circadian biology would hugely benefit from the development and deployment of functional proteomics methods. In addition to the initial steps of the gene expression program, protein abundance in the cell is regulated by translation, stability, and degradation mechanisms. Interestingly, a recent comprehensive study showed that protein abundance is not only determined by message abundance but that translation control is at least as important (Schwanhausser et al. 2011). The above-reviewed proteomics studies, though relatively limited, are consistent with a fundamental contribution of posttranscriptional regulation in the generation of daily rhythms. Therefore, comprehensive and quantitative analysis of the circadian behavior of the proteome will be a pre-requisite for a systematic and complete understanding of the function of circadian clocks in metabolism and physiology as well as for the effective application of this knowledge to pathologies associated with circadian rhythms such as sleep and metabolic disorders, cancer, etc (Barnard and Nolan 2008; Bass and Takahashi 2010; Huang et al. 2011; Takahashi et al. 2008).

As discuss above, recent advances in high-resolution MS quantitative proteomics allow quantification of the proteome and PTMs at a dramatically larger scale and depth. Therefore, we foresee that application of this technology to the circadian field will lead to promising outcomes (Fig. 2). First of all, the comprehensive analysis of global circadian changes of the proteome (protein levels and PTMs) in tissues and its comparison and complementation to the circadian variation of the transcriptome will result in a better understanding of the role of transcriptional and posttranscriptional regulation in the circadian clock and its relation to daily changes of behavior and physiology. Secondly, dynamic interaction proteomics of the molecular clock can delineate the temporal behavior of the complexes, uncovering the presence of novel protein interactions as well as the dynamics of functionally important PTMs in core clock proteins. Finally, spatial–temporal proteomics can lead to crucial information about the subcellular dynamics of clock protein complexes and its correlation to protein composition.

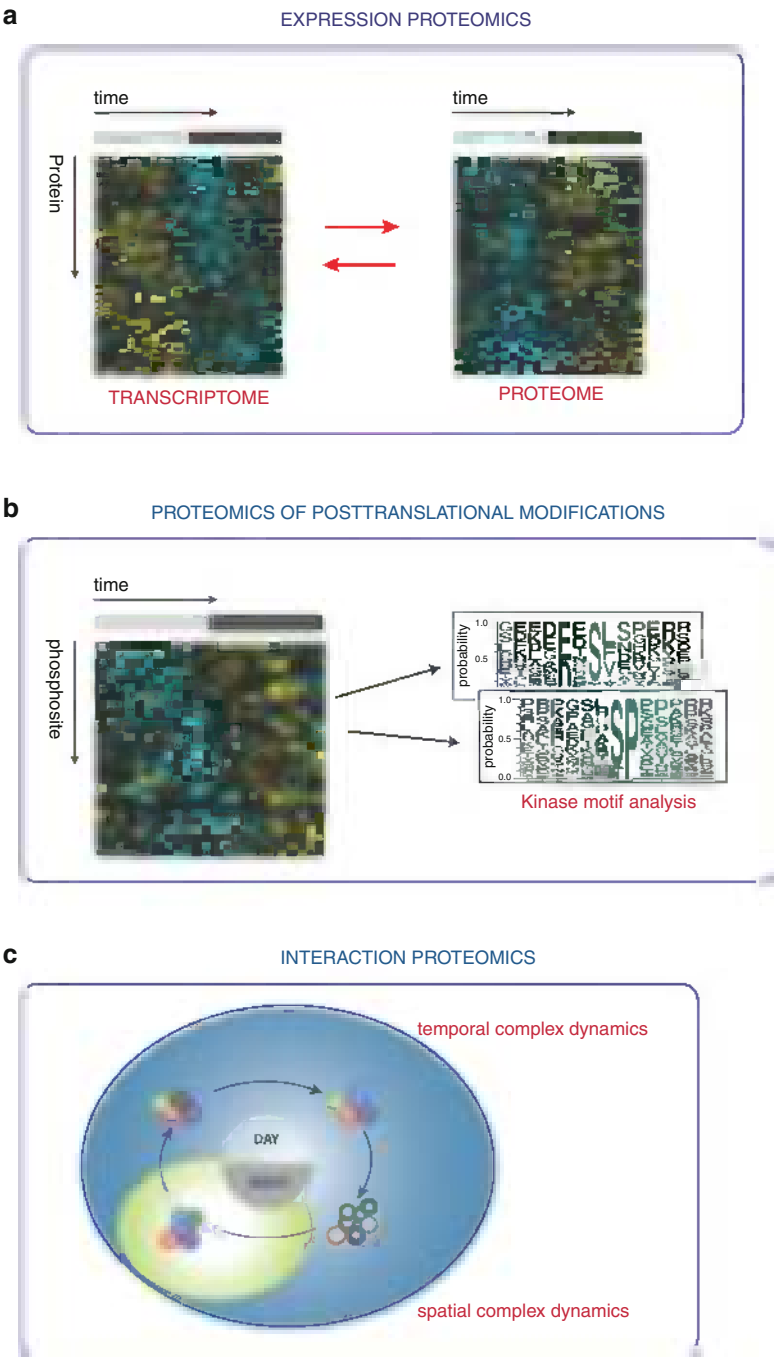

Fig. 2 Potential applications for high-resolution mass spectrometry based quantitative proteomics in the circadian field. (**a**) Expression proteomics application to the circadian clock could lead to

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Fig. 2 (continued) global temporal proteome datasets comparable in scale to previously reported transcriptomes. (**b**) Temporal quantification of PTMs, for example, phosphorylation, could generate large-scale data from which information about kinase activity could be retrieved. (**c**) Spatio-temporal interaction proteomics can also be applied to circadian biology to dissect dynamics and cellular localization of clock core complexes, both essential for proper clock function

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