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Michelle L. Gumz *Editor*

Circadian Clocks: Role in Health and Disease

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Circadian Clocks: Role in Health and Disease

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Contents

Chapter 1
Introduction to Circadian Rhythms and Mechanisms of Circadian Oscillations

David R. Weaver

Abstract Circadian rhythms are rhythms in behavior, physiology, or metabolism with a cycle length of approximately 24 h. Environmental disruption of daily rhythms leads to adverse health consequences, both in animal models and in humans engaged in shift work. The light–dark cycle has a major "entraining" influence on rhythms. Light for circadian entrainment is detected by rods, cones, and a population of intrinsically photosensitive retinal ganglion cells. The suprachiasmatic nuclei (SCN) generate and regulate circadian rhythms. SCN neurons coordinate their oscillations through several neurotransmitters and impact downstream neural structures by rhythmic neuronal firing. The molecular mechanism for circadian oscillation is a transcriptional–translational feedback loop that cycles with ~24 h periodicity in SCN neurons and also in other cell types. Many tissues maintain circadian oscillations even when isolated in vitro. Mechanisms by which the SCN coordinate these peripheral oscillators include rhythmic body temperature, food intake, and hormones. The molecular oscillator includes transcription factors, negative regulators, kinases, transcriptional co-activators and co-repressors, and posttranslational modifications to histone proteins. Several of these processes are influenced by metabolic factors. Several mutant mouse models are available for circadian rhythm research. While most data fit within the framework emphasized here, some "inconvenient truths" exist: under some circumstances, the SCN are not necessary for behavioral rhythms (with oscillations occurring by genetic mechanisms distinct from those discussed above), and in fact circadian oscillations can occur in the absence of transcription. Despite great progress in revealing circadian mechanisms over the past 20 years, much remains to be learned about circadian clocks.

Keywords Circadian • Diurnal • Rhythm • Rhythmicity • Feedback loop • Suprachiasmatic nucleus • Oscillator • Pacemaker • Gene expression • Time

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1.1 Introduction to Circadian Rhythms

The word circadian is derived from Latin terms meaning "approximately one day." Circadian rhythms are behavioral, physiological, and molecular rhythms with a cycle length of approximately 24 h. Figure 1.1a illustrates the locomotor activity rhythm of a normal mouse. Figure 1.1b illustrates locomotor activity rhythms recorded from a mouse with disruption of genes that cause the animal to lack circadian rhythmicity. These figures reveal key features of circadian rhythms, including the synchronizing effect of light–dark (LD) cycles, persistence of rhythmicity in constant darkness (DD; Fig. 1.1a), and "negative masking" by light leading to rhythmic behavior even in an animal that lacks ability to maintain rhythms in constant conditions (Fig. 1.1b). The presence of rhythmic behavior in the presence of a rhythmic environment (e.g., in a light–dark cycle in Fig. 1.1b) underlines the importance of determining if the rhythm is endogenously generated. Rhythms observed in a rhythmic environment are more properly called diurnal or daily rhythms. Environmental synchronizing signals, such as the light–dark cycle, are called "Zeitgebers" (German for "time-giver"), and time-points within such a cycle are referred to in units of Zeitgeber Time or ZT. By convention, in a 12L:12D lighting cycle, ZT0–ZT12 is the light phase (day), and ZT12–ZT24 is the dark phase (night).

To demonstrate that a rhythmic process is endogenous in nature (rather than reflecting responsiveness to a rhythmic environment), it must be observed under constant environmental conditions (e.g., constant darkness). In normal animals, rhythms typically persist in constant conditions with a species-typical periodicity that is near 24 h. Rhythms observed in constant conditions, free from the rhythmic environmental influences, are referred to as "free-running" circadian rhythms (even when the endpoint being examined is not running). In mice, the cycle length in constant darkness ("free-running period" or "tau_{DD}") is typically around 23.7 h, while in rats and humans it is approximately 24.2 h. Time-points within a cycle studied under constant conditions are referred to in units of Circadian Time (CT), which is determined either by extrapolation of the prior lighting cycle for 1–3 cycles, or by experimentally determining rhythm phase. In a nocturnal rodent, CT12 is defined as activity onset of the locomotor activity rhythm.

Locomotor activity is easily monitored and so is widely used as a measure of circadian rhythmicity, but this rhythm is just the tip of the iceberg. In mammals, almost all aspects of physiology vary with time of day, even under constant environmental conditions. The sleep/wake state, rate of cell division, renal potassium excretion, heart rate and blood pressure, immune and gastrointestinal function, sensitivity to drugs and toxins, and levels of many hormones (including melatonin and corticosteroids) all vary over the 24-h day, largely driven by alterations in gene expression in organs throughout the body. The implications of many of these physiological rhythms are discussed in the following chapters. The mechanisms by which these rhythms arise are discussed in detail below.

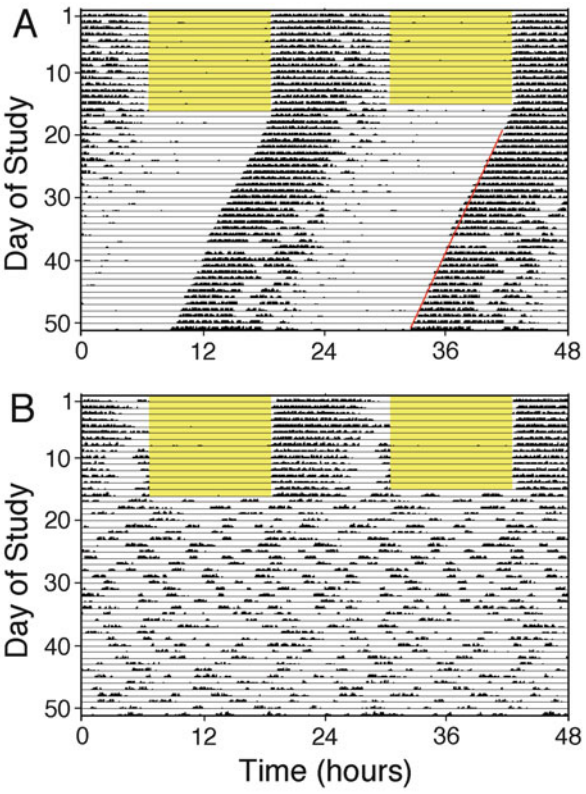

Fig. 1.1 Double-plotted activity plots (actograms) show several basic principles of circadian rhythms. In this plotting format, successive days of study are aligned on top of one another, and the data are artificially reproduced, and placed one day up and to the right, so that each row shows 48 continuous hours of data. Each animal is housed individually in a cage with a running wheel. Voluntary wheel-running activity is detected by magnets attached to the wheel. Bouts of activity appear in the plot as *dark bars*. *Yellow shading* indicates the times when lights are on. The animals were initially exposed to a 12 h light:12 h dark (12L:12D, or LD) lighting cycle for 3 weeks, and thereafter were exposed to constant darkness (DD). Panel (**a**) is an actogram from a wild-type mouse. When housed in LD, the mouse is active almost exclusively at night, as is typical of a nocturnal animal. Upon release into DD, the rhythmicity persists, but with a cycle length of slightly less than 24 h, so that activity onsets "drift" leftward (earlier) on successive days. (The typical "free-running period length" in constant darkness for C57BL/6J mice is ~23.7 h.) This actogram reveals that the mouse has an internal timekeeping system, with a cycle length near 24 h, and that light is normally synchronizing (resetting, or "entraining") the rhythm to the 24-h period of the lighting cycle. Panel (**b**) is an actogram from a mouse with a genetic mutation that leads to loss of circadian rhythmicity. The animal is active at night in LD, which appears normal. When transferred to DD, however, its locomotor activity is not rhythmic. The loss of rhythmicity in DD reveals that the rhythmicity observed in LD was in fact imposed by the LD cycle itself, rather than being due to an endogenous clock. This phenomenon ("negative masking") can confound interpretation of experiments and explains the need for many studies to be conducted in constant darkness

In addition to their endogenous nature (demonstrated by their persistence in constant conditions), another key feature of circadian rhythms is their ability to be synchronized to environmental perturbations. This property is essential for circadian clocks to be reset by environmental stimuli. Circadian oscillators are also temperature-compensated, meaning that the speed of the oscillation is remarkably unaffected by temperature (Q_{10} values are near 1.0; Dunlap et al. 2004). This feature makes the circadian oscillatory mechanism rather unusual, as the rate of most biochemical reactions is temperature dependent.

1.2 Physiological Significance of Circadian Rhythms

Circadian rhythms are widely thought to be an adaptive response to the 24-h cycles of light and dark, temperature, food availability, and predation that characterize our rotating world (Pittendrigh 1993; Dunlap et al. 2004; Vaze and Sharma 2013). Indeed, circadian rhythms are present in species ranging from cyanobacteria to *Homo sapiens*. The adaptive significance of circadian rhythms appears to stem from two sources, synchronization with the environment and internal synchronization. Synchronizing rhythms of the individual to the external environment likely optimizes resource acquisition while minimizing risks (e.g., animals forage for food in a temporal niche that minimizes exposure to their major predators). In addition, circadian rhythms lead to synchronization of physiological processes *within* the individual, even in constant conditions, a process sometimes referred to as "internal temporal order" (Moore-Ede et al. 1976). This synchronization optimizes physiological processes. For example, behaviors leading to food intake occur concurrently with the upregulation of salivary, gastric, pancreatic, hepatic, and intestinal genes that promote digestion and nutrient absorption (Scheving et al. 1983; Balakrishnan et al. 2012; see Chap. 10 by D. B. Rhoads). Thus, the circadian timing system provides *anticipation* of predictable environmental and physiological events and orchestrates *coordinated*, *temporally appropriate* changes in physiology. This is a critical concept, one that departs from the physiologist's usual understanding of homeostasis (in which a perturbation leads to corrective mechanisms that restore function to some defined set point). The circadian system actively modulates physiological set points over the 24-h day, providing predictive, rather than reactive, homeostasis (Moore-Ede 1986).

The most striking example of the importance of temporally coordinated, rhythmic processes among tissues is in the insect testis, where sequential sperm release, vas deferens acidification, and contractile activity are essential for fertility (Giebultowicz et al. 1989; Giebultowicz 2001; Beaver et al. 2002; Kotwica-Rolinska et al. 2013). Despite intense interest in the molecular and cellular mechanisms underlying circadian rhythm generation, relatively little attention has been paid to assessing the physiological importance of "internal temporal order." Recent advances allowing site-specific gene disruption in mice provide an inroad to assessing the importance of coordination among tissues.

1.3 Health Consequences of Circadian Rhythm Disruption

The ubiquitous nature of circadian rhythms and their ability to be affected by environmental perturbations has important consequences in our modern world. Most people are familiar with the disruption of their physiology that occurs when they fly across several time zones. The slow resynchronization to the new time zone leaves us with sleep disruption and gastrointestinal upset, among other consequences. Studies in mice suggest that jet lag is more than just an inconvenience, however. Animal studies employing "chronic jet lag" or shift work models to assess the impact of disrupting rhythms have shown adverse consequences for health and well-being (Evans and Davidson 2013). Circadian disruption increases mortality in aged mice (Davidson et al. 2006), impairs survival and immune function in young mice (Castanon-Cervantes et al. 2010; Park et al. 2012; Adams et al. 2013; Brager et al. 2013), promotes tumor growth (Filipski et al. 2004, 2009), impairs reproduction (Summa et al. 2012), promotes pancreatic β-cell loss, insulin resistance, and obesity (Gale et al. 2011; Lee et al. 2013a; Shi et al. 2013; Rakshit et al. 2014), promotes bowel inflammation and disease, alters the microbiome, and exacerbates alcohol-induced gut leakiness and hepatic pathology (Preuss et al. 2008; Summa et al. 2013; Yu et al. 2013; Voigt et al. 2014). The timing of food intake has a significant impact on metabolism and obesity (Arble et al. 2009, 2010; Salgado-Delgado et al. 2010; Hatori et al. 2012; Yoon et al. 2012; Garaulet et al. 2013), and there are numerous studies showing effects of environmental or genetic circadian disruption on metabolic endpoints and cross talk between metabolism and circadian mechanisms (Turek et al. 2005; Ramsey et al. 2009; Ruger and Scheer 2009; Marcheva et al. 2010; Kalsbeek et al. 2011; Karatsoreos et al. 2011; Paschos et al. 2012; Coomans et al. 2013a, b; Lee et al. 2013a; Shi et al. 2013). For reviews of the interplay between circadian clocks and metabolic processes, see Chap. 5 by C. B. Green, Arble et al. (2010), Asher and Schibler (2011), Aguilar-Arnal and Sassone-Corsi (2013), Peek et al. (2012). In addition, circadian disruption during pregnancy has long-lasting effects on the metabolic responses of the offspring (Varcoe et al. 2011, 2013).

These studies are of great interest in the context of understanding the health consequences of shift work in humans (Moore-Ede and Richardson 1985; Scott 2000; Gamble et al. 2013; Smith and Eastman 2013). Approximately, 10–20 % of workers in industrialized countries participate in shift work (working at times other than the day shift, including night workers and those on rotating shifts). Individuals participating in shift work often exist in a state of circadian disarray, sleeping, waking, and ingesting food at biologically inappropriate times (Ferguson et al. 2012). Shift work is associated with a higher incidence of a large number of maladies, from depression and mood disorders to metabolic syndrome, obesity, cardiovascular disease and stroke, autoimmune disorders, reproductive inefficiency, and cancer (Scott 2000; Straif et al. 2007; Suwazono et al. 2008a, b; Esquirol et al. 2009; Ruger and Scheer 2009; Antunes et al. 2010; Culpepper 2010; Evans and Davidson 2013; Gamble et al. 2013; Smith and Eastman 2013).

The International Agency for Research on Cancer concluded that "shift- work that involves circadian disruption is probably carcinogenic to humans" (Straif et al. 2007). With respect to metabolism, glucose ingested at a biologically inappropriate phase generates a poor insulin response that resembles a prediabetic state (Scheer et al. 2009), and sleep restriction with circadian disruption causes even more adverse metabolic responses (Buxton et al. 2012). Disruption of rhythms caused by irregularity in the lighting environment may be a factor contributing to the growing epidemic of obesity and metabolic syndrome. These studies suggest that the adverse health consequences of chronic circadian disruption are significant, as noted in more detail for specific organ systems and physiological functions in the following chapters of this volume.

"T-cycle" or "resonance" experiments are consistent with the idea that repeatedly adjusting your clock by large amounts has adverse consequences. In this type of experiment, the cycle length of the lighting cycle (T) is varied from 24 h, so that the circadian oscillator (which has its own preferred cycle length, *tau*) must readjust each day to remain synchronized. This readjustment occurs normally on a daily basis (e.g., to correct the 24.2 h period of our human circadian clock to the 24-h period of rotation of the Earth and to track the changing day length over the seasons), but this readjustment is in small increments. Repeated, large resynchronizing "phase shifts" have adverse consequences. In a variety of species from plants to cyanobacteria, from *Drosophila* to hamsters, agreement between the period of the internal clock and the external photocycle ("resonance") is beneficial (Pittendrigh and Minis 1972; Ooyang et al. 1998; Woelfle et al. 2004; Dodd et al. 2005; Martino et al. 2007, 2008; Vaze and Sharma 2013). (See Chap. 4 for more information on how disagreement between the internal clock and the external photocycle affect human physiology.) For example, when cyanobacterial strains with different endogenous period lengths were grown in competition, the "winner" in abundance was the strain in which the endogenous period more closely matched the period of the light:dark cycle (Ooyang et al. 1998; Woelfle et al. 2004). Martino and colleagues similarly found that mismatch between the period of the exogenous lighting cycle and the endogenous circadian clock produced adverse health outcomes, including cardiac and renal pathologies (Martino et al. 2007, 2008; for review, see Chap. 8 by T. Martino). Destruction of the suprachiasmatic nuclei (SCN), the brain region containing the master circadian clock, made the animals lose endogenous rhythmicity and also prevented the adverse effect of the mismatched lighting cycle; it appears worse to have a mismatched clock than to have no clock at all (Turek 2008).

1.4 Overview of the Mammalian Circadian Timing System

Biological timekeeping systems can be conceptually divided into three components (see Fig. 1.2a) (Eskin 1979). At the center of the system is a biological clock that measures time. A timekeeping mechanism is of little value if it cannot be adjusted

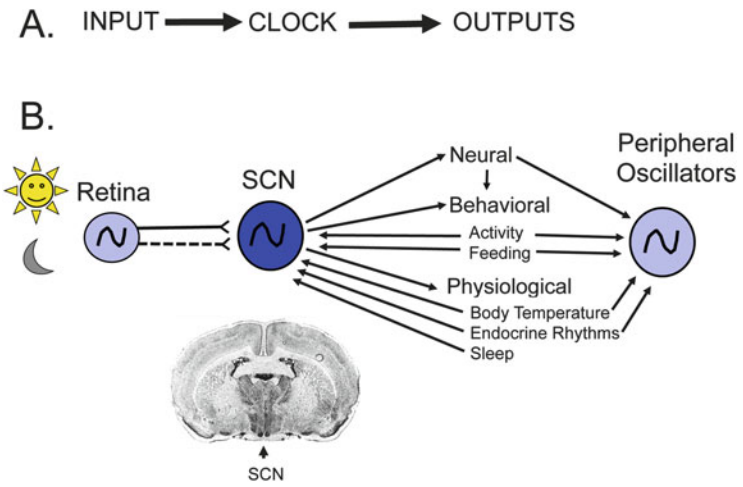

Fig. 1.2 The "Eskin-o-gram" method of illustrating a circadian system. (**a**) A circadian timing system can be conceptually broken down into input pathways that detect environmental signals and relay them to the clock, the oscillator (clock) itself, and output pathways through which the clock exerts its influence on physiological processes leading to overt rhythms in physiology, metabolism, and behavior. (**b**) The mammalian circadian timing system. Lighting conditions, detected by the retina, are relayed by direct and indirect pathways to the SCN in the anterior hypothalamus. The SCN (indicated by the *arrowhead* in the Nissl-stained section) is the primary circadian pacemaker. Through rhythmicity in its electrical firing rate and neurotransmitter release to other neural structures, the SCN regulates rhythms in many processes. The rhythms in food intake, body temperature, and corticosteroid and melatonin levels serve as reinforcing, entraining signals for oscillators located throughout the body ("peripheral oscillators")

or reset, and so input pathways are needed to detect and relay relevant environmental stimuli to the clock. Similarly, a biological clock is of little value if it does not engage physiological responses, and this occurs through output pathways leading to the overt rhythms we observe. In reality, the interactions among these components of the circadian timekeeping system in mammals are not unidirectional (e.g., overt rhythms can feed back to influence the biological clock), and the capacity for biological timekeeping is not limited to a single anatomical structure (Fig. 1.2b).

Circadian rhythms in mammals are controlled by a master circadian pacemaker in the suprachiasmatic nuclei (SCN) (see Weaver 1998; Welsh et al. 2010; Mohawk and Takahashi 2011; Weaver and Emery 2013) (Fig. 1.2b). The SCN are unique in having access to input from the retina critical for entrainment to light, and in their capacity to convey time of day information to other neural structures, and thereby to other tissues. Despite the dogmatic position to the contrary that predominated until the late 1990s, *the SCN are not unique in their capacity for molecular oscillations with a cycle length of ~24 h* (Balsalobre et al. 1998). Genes critical for expression of circadian rhythms are widely expressed, and many peripheral tissues display rhythmic gene expression in vivo and even in vitro (when explanted and cultured)

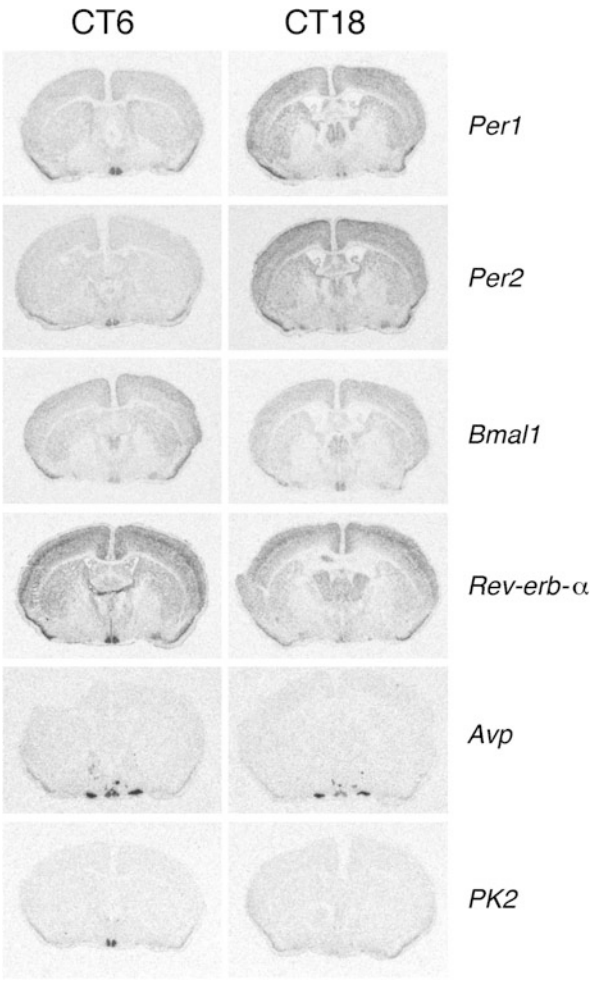

Fig. 1.3 Gene expression rhythms in the mouse SCN. Coronal sections through the mouse SCN were hybridized with ^{35}S-labeled antisense riboprobes to detect mRNAs: *Per1*, *Per2*, *Bmal1*, *Rev-erb-alpha*, *Avp*, and *Pk2*. Brain samples were collected on the first day in constant darkness, in the middle of the previous light period (CT6, *left column*), or the middle of the previous dark period (CT18, *right column*). Each of the transcripts is rhythmic in the SCN. All transcripts except *Bmal1* are higher levels at CT6 than at CT18. *Bmal1* mRNA is higher at CT18 than at CT6. Images are from experiments conducted by Jason DeBruyne and Chris Lambert and reported in DeBruyne et al. (2006)

(Tosini and Menaker 1996; Yamazaki et al. 2000; Abe et al. 2002; for reviews see Dibner et al. 2010; Mohawk et al. 2012; Menaker et al. 2013) (Fig. 1.3).

If almost all cells contain the capacity for cell-autonomous circadian oscillations, why are the SCN held in such special regard within the circadian field? The answer lies in the distinction between an oscillator and a pacemaker. SCN neurons communicate with each other, leading to a tissue-level rhythm that can persist in vitro. SCN explants can oscillate for months, even years, in vitro (see Yamazaki and Takahashi 2005). But the SCN do more than oscillate. The SCN convey their rhythmicity along neuroanatomical output pathways, leading to physiological and behavioral rhythms that coordinate rhythms within and between peripheral tissues, serving as a pacemaker. The mammalian circadian timing system is hierarchical: non-SCN "peripheral oscillators" are synchronized by a variety of SCN-regulated output rhythms including body temperature, corticosteroid levels, melatonin levels,

food intake, and direct autonomic innervation (Balsalobre et al. 2000; Damiola et al. 2000; Brown et al. 2002; von Gall et al. 2002; Buhr et al. 2010; Saini et al. 2012, 2013; Gerber et al. 2013; for a review see Mohawk et al. 2012; Menaker et al. 2013; Wood and Loudon 2014). Blood-borne signals entrain peripheral tissues in part through Serum Response Factor-1 (Gerber et al. 2013), while Poly (ADP-ribose) polymerase 1 is involved in food entrainment of the liver oscillator (Asher et al. 2010). In the absence of the SCN, behavioral rhythms and most physiological rhythms are lost, but there is evidence for persistence of some tissue-level oscillations (Yoo et al. 2004; Tahara et al. 2012; Saini et al. 2013). Thus, coordinating the activity of circadian oscillators in peripheral tissues is a key role of the SCN in maintaining internal temporal order (Yoo et al. 2004, 2005; Mohawk et al. 2012; Tahara et al. 2012; Menaker et al. 2013; Saini et al. 2013).

The following sections will discuss the input role of the retina and the pacemaker role of the SCN and include a brief discussion of output mechanisms.

1.4.1 Input: Circadian Photoreception by the Retina

The most influential environmental stimulus affecting circadian rhythms is the daily light–dark cycle. In mammals, information about the environmental light–dark cycle leading to synchronization of circadian rhythms (entrainment) is detected in the retina. Eye removal makes the mammalian circadian system completely blind to light (Yamazaki et al. 1999). Destruction of rods and cones, or blocking opsin-based signaling in these classical photoreceptors, prevents visual image formation but, remarkably, does *not* prevent circadian photoreception (Provencio et al. 1994; Lucas et al. 1999; Freedman et al. 1999). A specialized population of intrinsically photosensitive retinal ganglion cells (ipRGCs) expresses the photoreceptive molecule, melanopsin (Berson et al. 2002; Hattar et al. 2002; Provencio et al. 2002). Mice lacking melanopsin lose the intrinsic light sensitivity in their retinal ganglion cells but maintain the ability to entrain to lighting cycles (Panda et al. 2002b; Ruby et al. 2002). In melanopsin-deficient mice, phase shifts of circadian rhythms in response to brief light exposure and the light-induced pupillary constriction are preserved, but are markedly attenuated and require higher light intensities (Panda et al. 2002b; Ruby et al. 2002; Gooley et al. 2003; Lucas et al. 2003). The ipRGCs receive input from the classical photoreceptors (the rods and cones, via amacrine and bipolar cells), and the classical photoreceptors can signal through the ipRGCs even in the absence of melanopsin. Destruction of the ipRGCs, or blocking rod and cone signaling in melanopsin-deficient mice, prevents circadian light perception (Hattar et al. 2003; Panda et al. 2003; Guler et al. 2008; Hatori et al. 2008). For reviews discussing circadian photoreception and ipRGCs, see Do and Yau (2010), Schmidt et al. (2011) and Lucas (2013).

1.4.2 *The Master Circadian Pacemaker Is Located in the Suprachiasmatic Nuclei*

A major projection site of the ipRGCs is the suprachiasmatic nuclei (SCN) (Gooley et al. 2001; Hattar et al. 2002). These small, oval nuclei sit just dorsal to the optic chiasm in the anterior hypothalamus (Figs. 1.2b and 1.4). Several lines of evidence define the SCN as the master circadian pacemaker in mammals (see Weaver 1998). Studies dating back to the early 1970s showed that destruction of the SCN leads to loss of behavioral and physiological circadian rhythms. Transplantation of tissue containing the fetal SCN restores circadian rhythmicity in SCN-lesioned, arrhythmic hosts, and the cycle length of rhythmicity is dictated by the transplanted tissue rather than by host genotype (Ralph et al. 1990). Thus, the SCN are, in most cases, necessary and sufficient for overt circadian rhythms in rodents (See Sect. 1.7 for an explanation of "in most cases").

The SCN contain approximately 20,000 neurons in mice. These neurons are heterogeneous with respect to neuropeptide phenotype, connectivity, and other properties (Welsh et al. 2010; Mohawk and Takahashi 2011; Fig. 1.5). Most SCN neurons have the capacity for cell-autonomous rhythmicity, as demonstrated by recording of electrical activity or bioluminescence rhythms from individual SCN cells when neurons are dispersed so that they lack functional connectivity (Welsh

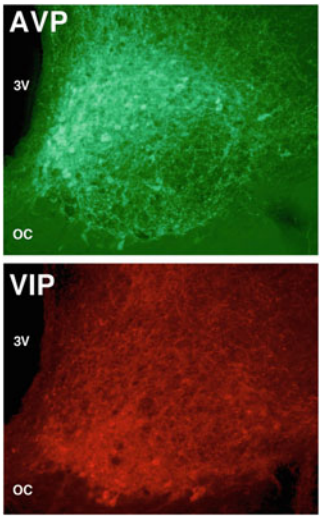

Fig. 1.4 Neuropeptides define subregions of the rat SCN. The images show immunofluorescence staining of adjacent sections in the rat SCN. Each panel shows a unilateral SCN. The *upper panel* shows the localization of AVP; cell bodies are located in the dorsal and medial regions of the SCN, with fiber staining throughout the nucleus. The *lower panel* shows VIP; cell bodies are located primarily in the ventral SCN, and VIP-positive fibers extend throughout the nucleus. The third ventricle (3V) is at the midline, and the optic chiasm (OC) encompasses the ventral border of the SCN. Images courtesy of Matthew J. Paul and William J. Schwartz

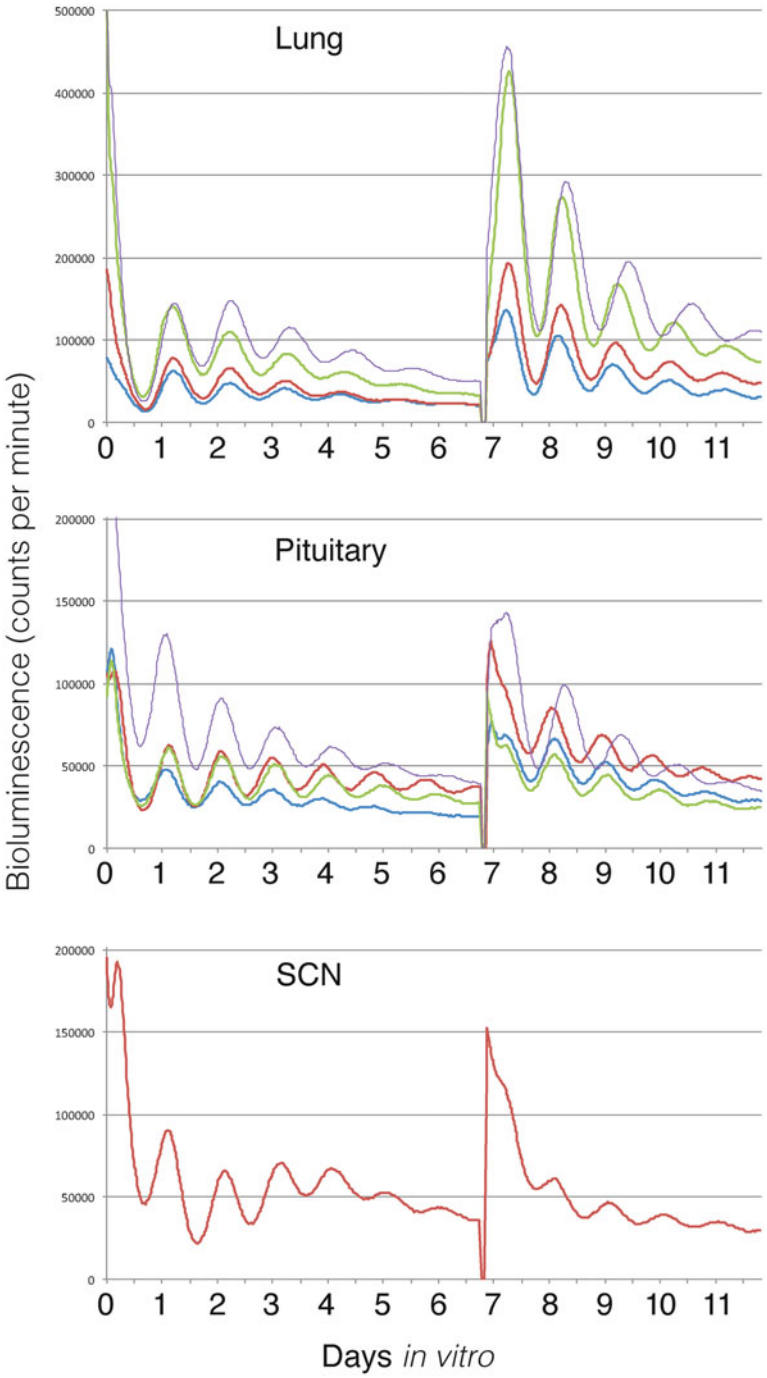

Fig. 1.5 Bioluminescence traces from tissue explants. Explants of lung (*top*), pituitary (*center*), and SCN (*bottom*) from a Per2::luciferase reporter mouse were maintained in vitro in the presence of luciferin (substrate), and rhythms of bioluminescence were recorded at 15-min intervals with a

et al. 1995; Liu et al. 2007a). When the SCN are intact, the multiple single-cell oscillators communicate to synchronize to a single cycle length, establishing spatiotemporal waves of gene expression in which subpopulations of neurons are coupled at different phase relationships to each other (Evans et al. 2013).

1.4.2.1 Neurochemical Interactions Among SCN Neurons

SCN neurons interact by neurochemical mechanisms to coordinate their activity, generating coherent molecular and firing rate rhythms, and transmitting this rhythm via neuronal connections to output pathways regulating overt physiological and behavioral rhythms. Intercellular coupling within the SCN is key for circadian clock function (see Mohawk and Takahashi 2011). Several neurotransmitters, including vasoactive intestinal peptide (VIP), gastrin-releasing peptide (GRP), and gamma amino butyric acid (GABA), are important for interactions among SCN neurons (Maywood et al. 2011). VIP is expressed in a subset of neurons in the central "core" region of the SCN. VIP-containing neurons project heavily within the SCN core, to the surrounding "shell" region, and also beyond the SCN borders. Mice lacking VIP, or the VPAC2 receptor for VIP, have severely disrupted circadian rhythms (Colwell et al. 2003; Aton et al. 2005; Maywood et al. 2006). At the cellular level, the amplitude of individual neuronal oscillators is reduced in the absence of VIP or VPAC2 signaling, and the neurons are not coordinated to each other (Aton et al. 2005; Maywood et al. 2006). These findings strongly suggest that a "coupling deficit" leads to altered circadian rhythmicity in these lines of mice. VIP/VPAC2 acts through elevation of cAMP levels in SCN (Atkinson et al. 2011). Indeed, activation of intracellular signal transduction pathways seems important for maintenance of SCN neuronal synchronization (O'Neill et al. 2008; Brancaccio et al. 2013). In *Drosophila*, peptide dispersing hormone (PDF) is the homolog of VIP, and PDF and its receptor play a similar role to mammalian VIP and the VPAC2 receptor in coordinating neurons within the fly circadian neural network (Duvall and Taghert 2013).

Fig. 1.5 (continued) photomultiplier tube within the incubator. Raw bioluminescence counts per minute (cpm) are plotted against time in vitro. These data illustrate the presence of circadian oscillators in peripheral tissues, as well as in the SCN. Imaging studies reveal that the loss of rhythm amplitude that occurs over time in culture is due to loss of synchronization among the many single-cell oscillators in the population, rather than loss of rhythms per se (e.g., Nagoshi et al. 2004; Leise et al. 2012). This interpretation is supported by the reinstatement of robust rhythmicity by 1-h exposure to a resetting stimulus (100 nM dexamethasone) on day 7 of the record

1.4.2.2 Regulation of SCN Neuronal Excitability and Firing Rate

SCN neurons have a cell-autonomous circadian clock, with multiple mechanisms contributing to rhythmicity in SCN ion channel activity, SCN neuronal firing rate, and SCN neurotransmitter release. Rhythmicity of SCN potassium channel activity appears critical for producing high-amplitude circadian rhythms in membrane potential and SCN firing rate (Itri et al. 2005; Meredith et al. 2006; Kudo et al. 2011; Hablitz et al. 2014). Disruption of rhythmic potassium channel activity (either large conductance "BK" channels or fast delayed rectifier current) leads to loss of rhythm amplitude. This is likely due to a reduced ability of individual SCN neurons to generate robust, cell-autonomous oscillations, and secondarily of SCN neurons to regulate neurotransmitter release. G protein-coupled inwardly rectifying potassium channels (GIRKs) are also rhythmically expressed and appear to play an important role in mediating phase shifts by non-photic stimuli (Hablitz et al. 2014).

Sodium-dependent action potentials are important for synchronizing SCN neurons (Yamaguchi et al. 2003). This may be due to the critical role of action potentials in synaptic release of SCN neurotransmitters, including VIP, GRP, and GABA. Synaptic vesicle cycling also plays an important role in the regulation of circadian rhythms; several synaptic vesicle proteins are important for circadian rhythmicity (Kapfhamer et al. 2002; Deery et al. 2009; Kim et al. 2009; Punia et al. 2012).

1.4.2.3 Transcription Factors Controlling Neuropeptide Expression Are Important for Circadian Rhythmicity

The transcription factor LHX1 was found to be important for SCN development, and in the adult, also important for expression of several SCN neuropeptides including VIP and GRP (Bedont et al. 2014; Hatori et al. 2014). Mice lacking LHX1 in the SCN, either throughout life or in adulthood, have severely disrupted circadian rhythms, likely due to disruption of SCN neuronal synchronization (Bedont et al. 2014; Hatori et al. 2014). Similarly, Parsons et al. (2015) reported that a transcription factor called zinc finger homeobox 3 (*Zfhx3*) is the gene mutated in the *Short-circuit* (*Sci*) mutant line, which has abnormal circadian rhythms (Parsons et al. 2015). ZFHX3 appears important for expression of several SCN neuropeptides.

1.4.3 SCN Outputs

Anatomically, the major projection of the SCN emerges caudally and dorsally, innervating the subparaventricular zone (SPZ) extensively (for reviews, see Saper et al. 2005; Weaver and Emery 2013). The SPZ in turn projects to many brain areas,

and these brain areas often receive a converging, direct input from the SCN. The net effect of rhythmic neuronal activity in these output pathways is the generation of overt behavioral, physiological, and hormonal rhythms. These output rhythms, in turn, lead to synchronization of circadian oscillators in non-SCN sites in brain and in peripheral tissues (Mohawk et al. 2012; Menaker et al. 2013). GABA, glutamate, and arginine vasopressin (AVP) are among the neurotransmitters expressed in SCN neurons and shown to be important for controlling output functions (Jin et al. 1999; Kalsbeek et al. 2006).

The most well-understood output pathway is the one by which the SCN regulate pineal arylalkylamine *N*-acetyltransferase (AANAT) activity, the rate-limiting step in melatonin production. This first step in this pathway involves GABAergic output from the SCN, which inhibits the paraventricular hypothalamus during the daytime (Kalsbeek et al. 2006). At night, when SCN neuronal activity is lower, the PVN is disinhibited and melatonin production occurs. The pathway from the PVN to the pineal relays in the intermediolateral cell column of the spinal cord, from which preganglionic autonomic fibers innervate the superior cervical ganglion. The final step is axons from the superior cervical ganglion, which reenter the cranium and innervate the pineal gland. Norepinephrine released from these terminals increases AANAT mRNA in rodents and also protects the enzyme by inhibiting proteasomal degradation (Gastel et al. 1998). Light exposure at night leads to rapid proteasomal degradation of AANAT protein, leading to serum melatonin reaching minimum values within minutes.

Other output pathways are less well described. One area that has received considerable attention is the mechanisms by which the SCN regulates locomotor activity. Several neuropeptides are thought to be important for regulating locomotor activity through their release from the SCN, either locally or into the cerebrospinal fluid. This line of study was prompted by a study by Rae Silver and colleagues (1996), demonstrating that SCN transplants encapsulated in permeable membranes were nevertheless effective in restoring rhythmicity in SCN-lesioned hosts. These membranes allowed diffusible substances to be secreted from the SCN but prevented axonal outgrowth, indicating that humoral factors from the SCN were capable of rhythm restoration.

Weitz and colleagues conducted a screen to identify SCN-expressed peptides that suppress locomotor activity. Transforming growth factor alpha (TGF-α) and cardiotropin-like cytokine 1 were identified as SCN peptides that suppress locomotor activity (Kramer et al. 2001; Kraves and Weitz 2006). Snodgrass-Belt et al. (2005) found that additional behaviors were disrupted by TGF-α infusion, however, and that additional cytokines and their receptors could inhibit rhythmically expressed behaviors. The action of these cytokines may be more related to fatigue (Harrington 2012) than to the circadian regulation of locomotor activity.

Cheng et al. (2002) proposed a similar activity-suppressing role for prokineticin 2 (PK2). In mice, the rhythm of PK2 mRNA expression peaks several hours after lights-on (or several hours after the projected time of lights-on in animals kept in constant dark). Administration of PK2 by intracerebroventricular injection at night inhibited locomotor activity (Cheng et al. 2002). This fits with its proposed role in

mice: PK2 levels are high during the day when the animals are inactive, while PK2 levels are low at night, "allowing" activity at night. Curiously, however, day-active Nile grass rats have a similar daytime peak PK2 expression in the SCN (Lambert et al. 2005). Peak PK2 expression occurring when the grass rats are active seems inconsistent with the proposal that PK2 is a critical activity-suppressing output from the SCN. In humans, loss of PK2 in Kallmann syndrome does not disrupt central circadian oscillator function or physiological rhythmicity (Balasubramanian et al. 2014). PK2 may play roles within the circadian clock, rather than as an output molecule acutely regulating locomotor activity (Li et al. 2012; Bedont et al. 2014).

As noted earlier, the SCN generate physiological rhythms (food intake, body temperature, and hormone levels) that in turn entrain the endogenous rhythmicity of oscillators in other organs. The extent and importance of the rhythmicity in peripheral tissues has only recently become appreciated. Microarray studies of liver, heart, intestine, retina, and other tissues reveal that >10 % of expressed genes are rhythmically expressed in each tissue (Akhtar et al. 2002; Panda et al. 2002a; Storch et al. 2002; Oishi et al. 2003). Rhythmically expressed genes tend to be key, rate-limiting steps regulating tissue-specific biological pathways. Both transcriptional and post-transcriptional mechanisms contribute to rhythmicity of gene and protein expression (see Sect. 1.5; Koike et al. 2012; Menet et al. 2012; Lim and Allada 2013b; Kojima and Green 2015).

A well-recognized and clinically relevant indication of rhythmicity in peripheral tissues is the finding that both therapeutic and toxic side effects of many drugs are dependent on the time of day of administration. Indeed, the field of chronopharmacology/chronotherapeutics seeks to understand rhythms in drug disposition and efficacy and to optimize the therapeutic index by defining optimum times of drug administration [Gachon and Firsov 2011; Dallmann et al. 2014; Chap. 11 (Levi); Chap. 12 (Hermida)]. The contribution of the molecular clock to rhythmic drug effects was examined by DeBruyne et al. (2014), who showed that tissue-autonomous clocks in the liver influence the rate of pentobarbital metabolism (affecting the duration of anesthesia) and the level of acetaminophen toxicity (due to time-dependent differences in its metabolism to a toxic metabolite). Hepatic metabolism and renal excretion figure prominently in chronotherapeutics, but variations in target-tissue sensitivity also exist (cf. Kino and Chrousos 2011).

1.5 The Molecular Mechanisms Underlying Circadian Oscillation

1.5.1 Overview

As noted above, single cells can express circadian rhythms. The intracellular mechanism of oscillation is a transcriptional–translational feedback loop (see Fig. 1.6). In broad terms, activating transcription factors promote the expression of genes whose products feed back to inhibit their own production, forming a

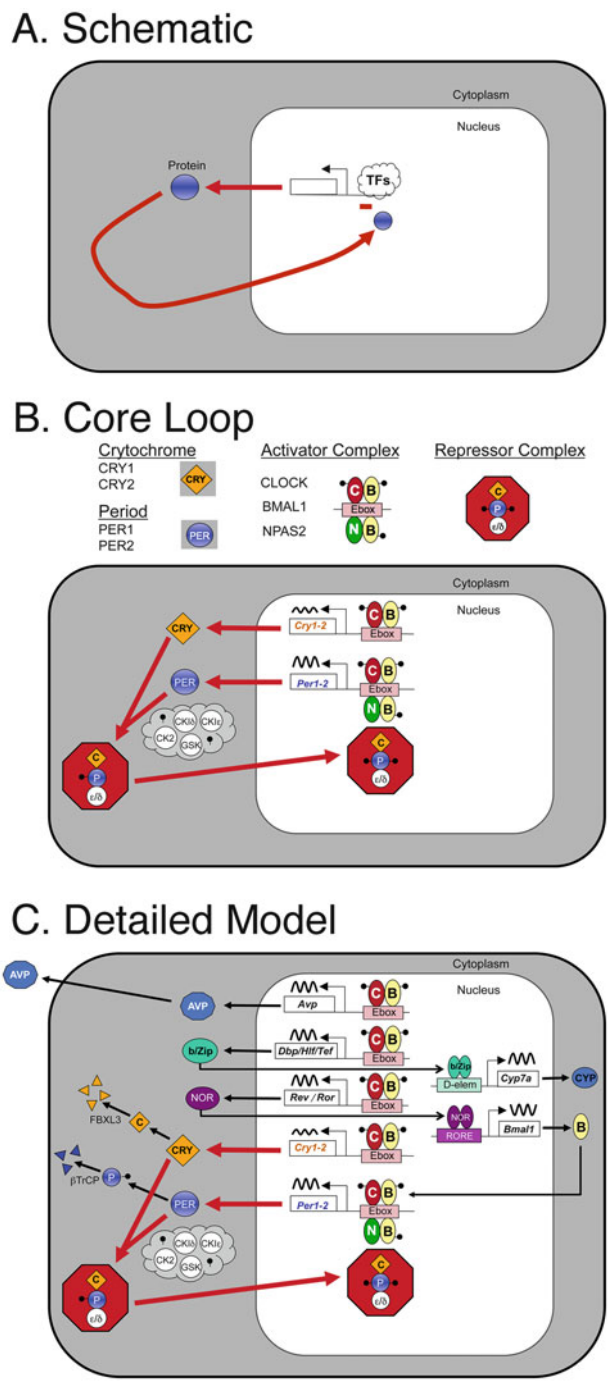

Fig. 1.6 The core circadian feedback loop. The core clock loop establishes concentric waves of temporally regulated, tissue-specific gene expression. Panel (**a**) shows generic transcriptional/translational feedback loop, in which Transcription factors (TFs) bind to promoter elements of

feedback loop with a cycle length of approximately 24 h (Fig. 1.6a). The mRNAs and proteins that provide the most dynamic regulation of the clock are produced and then broken down within each circadian cycle. There are multiple, parallel, and interlocking feedback loops that together reinforce rhythmicity, refine cycle length, and augment rhythm amplitude. The molecules and mechanisms for circadian rhythms are well conserved between flies and mammals (Weaver and Emery 2013). Below, I will describe the components of the mammalian core feedback loop (for reviews, see Chong et al. 2012; Partch et al. 2014).

1.5.2 Activation Phase

In mammals, the transcriptional activator complex consists of heterodimers containing basic helix-loop-helix (bHLH) and Per-ARNT-SIM (PAS) domains (Fig. 1.6b). The bHLH-PAS proteins CLOCK and BMAL1 form a heterodimeric complex that is central to circadian oscillator function (BMAL1 is also called MOP3 and ARNTL) (see Table 1.1 for a list of gene synonyms). In a subset of neurons in the SCN, neuronal PAS domain-containing 2 (NPAS2, a bHLH-PAS protein with high sequence homology to CLOCK) is expressed and can maintain circadian rhythms even in the absence of CLOCK, presumably by interacting with and stabilizing BMAL1 (DeBruyne et al. 2007a). Mice lacking BMAL1 or lacking both of BMAL1's heterodimeric partners (CLOCK and NPAS2) lack circadian rhythms (Bunger et al. 2000; DeBruyne et al. 2007a). In peripheral tissues, NPAS2 is expressed at low levels and so cannot compensate for the absence of CLOCK (DeBruyne et al. 2007b). NPAS2 contributes to molecular oscillations in forebrain

Fig. 1.6 (continued) responsive genes, leading to transcription of mRNAs, translation of the protein, and subsequent negative feedback by the products to repress their own production. Panel (**b**) shows the core circadian feedback loop in mammals. The transcriptional activation complexes consist of heterodimers of CLOCK and BMAL1, and/or NPAS2 and BMAL1. The activator complex drives the rhythmic expression of two *Period* and two *Cryptochrome* genes. The PER and CRY protein products are subjected to phosphorylation by casein kinase 1 delta (CK1 δ), casein kinase 1 ε (CK1e), casein kinase 2 (CK2), and GSK-3beta, which influence stability and their functional properties. Multiprotein complexes effect repression by interfering with the transcriptional activation complex. Panel (**c**) illustrates a more extended version of the feedback mechanism, indicating how tissue- and phase-specific gene expression patterns occur. Secondary loops include nuclear orphan receptors (NORs) and the members of the DBP/HLF/TEF/E4BP4 family. These transcription factors are controlled by the CLOCK/NPAS2:BMAL1 complex, and influence the feedback loop, in addition to driving their own response genes in a tissue-specific manner. For example, in liver cells, the DBP family (and opposing E4BP4) regulate Cyp gene expression, influencing bile acid production. A target of the nuclear orphan receptor loop is *Bmal1*, with the predominant effect being repression by REV-ERB-alpha, so that *Bmal1* levels are suppressed during subjective day (see Fig. 1.3). In SCN neurons, arginine vasopressin (AVP) is a directly clock-controlled gene. Rhythmic release of AVP into brain regions and into cerebrospinal fluid influence physiological responses

Table 1.1 Core circadian "clock gene" products and other clock-relevant protein families

Abbreviation	Full name	Common alternative names
bHLH-PAS proteins		
CLOCK	Circadian locomotor output cycles kaput	bHLHe8
NPAS2	Neuronal PAS domain protein 2	Mop4, bHLHe9
BMAL1	Brain and muscle ARNT-like 1	Arntl, bHLHe5, Mop3, Arnt3
BMAL2	Brain and muscle ARNT-like 2	Arntl2, bHLHe6, Mop9, Clif
DEC1	Differentiated embryonic chondrocyte-expressed gene 1	Bhlhe40, Sharp-2, Stra13
DEC2	Differentiated embryonic chondrocyte-expressed gene 2	Bhlhe41, Sharp-1
PERIOD proteins		
PER1	Period1 (period circadian protein homolog 1)	
PER2	Period2 (period circadian protein homolog 2)	
PER3	Period3 (period circadian protein homolog 3)	
Cryptochromes		
CRY1	Cryptochrome 1 (photolyase-like)	
CRY2	Cryptochrome 2 (photolyase-like)	
Orphan nuclear receptors		
REV-ERBα	Nuclear receptor subfamily 1, group D, member 1	Nr1d1, Rev-erb-alpha
REV-ERBβ	Nuclear receptor subfamily 1, group D, member 2	Nr1d2, Rev-erb-beta, RVR
RORα	RAR-related orphan receptor alpha (ROR-alpha)	Nr1f1, Rora, ROR1
RORβ	RAR-related orphan receptor beta (ROR-beta)	Nr1f2, Rorb, RZR-beta
RORγ	RAR-related orphan receptor gamma (ROR-gamma)	Nr1f3, Rorc, Thor, TOR
Casein kinases		
CK1δ	Casein kinase 1 delta	Csnk1d, CK1d
CK1ε	Casein kinase 1 epsilon	Csnk1e, CK1e, *tau* mutation
CK1α	Casein kinase 1 alpha	Csnk1a1
CK2	Casein kinase 2 alpha 1	Csnk2a1
Ubiquitin ligase (SCF complex) F box proteins		
FBXL3	F-box and leucine-rich repeat protein 3	SCF F-box like 3
FBXL21	F-box and leucine-rich repeat protein 21	SCFFbxl21, FBXL3B
β-TrCP1	beta-transducin repeat containing protein	Fbw1a; FWD1
β-TrCP2	beta-transducin repeat-containing protein 2	Fbxw11; BTRCP
PAR bZIP transcription factors		
DBP	D site albumin promoter binding protein	D-box binding protein
HLF	Hepatic leukemia factor	
TEF	Thyrotroph embryonic factor	
E4BP4	E4 promoter-binding protein 4	Nuclear factor IL3 (NFIL3)

(Reick et al. 2001). Because the role of NPAS2 has not been as extensively characterized, and for simplicity, I will focus on discussing the CLOCK:BMAL1 complex while expecting that the role of the NPAS2:BMAL1 may be similar in areas where NPAS2 is expressed. The CLOCK:BMAL1 heterodimers interact with specific hexanucleotide DNA sequences (E and E′ boxes) to enhance transcription of the genes that feed back to close the loop, as well as other genes (see below). Several studies have identified many direct transcriptional targets of CLOCK:BMAL1 complexes (Hatanaka et al. 2010; Rey et al. 2011; Guillaumond et al. 2012; Koike et al. 2012; Menet et al. 2012; Shimomura et al. 2013).

1.5.3 Repression Phase

Among the genes regulated by the CLOCK:BMAL1 complex are the three *Period* genes (*Per1*, *Per2*, and *Per3*) and two *Cryptochrome* genes (*Cry1* and *Cry2*). (The convention used in this chapter and by most journals is that the gene name is italicized, and the protein product is in all-capitalized, non-italicized font as shown in the next sentence.) The protein product of the *Per3* gene, PER3, seems to have at most a modest contribution to circadian regulation (Shearman et al. 2000). In contrast, PER1 and PER2 are critically important. There is partial redundancy of function between PER1 and PER2 and between CRY1 and CRY2. Deletion of both *Per1* and *Per2*, or of both *Cry1* and *Cry2*, leads to loss of circadian rhythmicity (van der Horst et al. 1999; Vitaterna et al. 1999; Zheng et al. 2001; Bae et al. 2001). The PER and CRY proteins form complexes essential for negative feedback. The high-amplitude rhythmic expression of PER1 and PER2 is the rate-limiting step for the formation of PER:CRY complexes (Lee et al. 2001). Constitutive expression of CRY does not disrupt oscillator function, while constitutive expression of PER does. Nevertheless, regulation of the stability of both PERs and CRYs influences the time interval between transcriptional activation and negative feedback and thus the period of the circadian oscillation (see Sect. 1.5.6).

1.5.4 Transcription Factor Cascades: Like Ripples on a Pond

In addition to the genes involved in the core feedback loop, many other genes are rhythmically expressed in a circadian-clock dependent manner. As noted earlier, ~10 % of expressed genes are rhythmically expressed in each of the tissues examined to date, and the rhythmically expressed genes tend to be key, rate-limiting steps (Akhtar et al. 2002; Panda et al. 2002a; Storch et al. 2002; Oishi et al. 2003).

Some clock-controlled genes are controlled directly by CLOCK/NPAS2:BMAL1 complexes in parallel with the PER and CRY genes, but do not participate in the feedback loop or in transcriptional networks (Fig. 1.6c). Arginine vasopressin (AVP) in the SCN is a premier example of an output gene directly regulated at the transcriptional level by the CLOCK:BMAL1 complex (Jin et al. 1999). This

neuropeptide is rhythmically released at neuronal sites and into the cerebrospinal fluid, influencing brain function, but without being part of a molecular feedback loop affecting its own regulation.

Other genes regulated by CLOCK/NPAS2:BMAL1 heterodimers are themselves transcription factors. They can be either transcriptional activators or transcriptional repressors. Members of four transcription factor families (nuclear hormone receptors, PAR b-Zip, DEC1/DEC2, and Chrono) are believed to form secondary, interlocking feedback loops. These interlocking loops appear to stabilize and reinforce the primary CLOCK/NPAS2:BMAL1 → PER1/PER2:CRY1/CRY2 feedback mechanism. Similar secondary loops exist in *Drosophila*, and some of the proteins involved are homologous to their mammalian counterparts. An interesting feature of these transcription factor networks is that the activator complex often turns on expression of genes, within the same gene family, that have opposing activities. The competition and antagonism between activators and repressors for promoter elements appears to be an important and conserved design element in the circadian clock.

The transcription factors of the interlocking feedback loops affect the three major response elements that contribute to rhythmic transcription: E- and E-like boxes (responsive to bHLH-PAS proteins and CHRONO), D-box elements (responsive to the PAR bZIP family), and RORE elements (responsive to orphan nuclear hormone receptors) (Ueda et al. 2005). The rhythmic expression of these transcriptional factor cascades leads, with some delay, to regulation of the expression of second-order genes responsive to their activity. Some of these second-order clock-controlled genes are, again, transcription factors. Thus, like ripples spreading outward from a pebble thrown into a pond, CLOCK/NPAS2:BMAL1 heterodimers initiate ever-widening waves of gene expression, achieving a variety of phases and profiles of gene expression. Post-transcriptional mechanisms provide even further regulation (see Sect. 1.5.9).

1.5.5 Interlocking Feedback Loops

As noted above, four protein families have been identified that form interlocking feedback loops that operate in parallel with the CLOCK:BMAL1 → PER:CRY core feedback loop. These modifiers are generally dispensable for circadian oscillation, but can have important roles in modifying gene expression rhythm amplitude and phase.

Several nuclear orphan receptors (Reverb-α, ROR-α, and ROR-γ-t) are examples of CLOCK/NPAS2:BMAL1 targets that regulate other genes. Among the genes regulated by the nuclear orphan family members, acting upon nuclear orphan receptor response elements is *Bmal1*. The RORs activate, while REV-ERB-α represses, *Bmal1* gene expression, forming an interlocking feedback loop within the circadian clock (Preitner et al. 2002; Ueda et al. 2002, 2005; Sato et al. 2004). Constitutive overexpression of REVERB-α depresses *Bmal1* expression;

Kornmann et al. (2007) used liver-specific overexpression of Reverb-α to suppress BMAL1 levels and thus disrupt circadian clock function in liver, showing the importance of this regulatory mechanism. The nuclear hormone receptor family is discussed at greater length in Chap. 13 (Burris).

A family of "proline- and acidic amino acid-rich basic leucine zipper proteins," the PAR bZIP transcription factors, participate in another interlocking feedback loop (Gachon 2007). These PAR bZIP transcription factors include the activators DBP (D-box binding protein), TEF (thyrotroph embryonic factor), HLF (hepatic leukemia factor), and the repressor E4BP4 (E4 promoter-binding protein 4). Expression of these genes is increased by CLOCK:BMAL1 complexes, and they are rhythmically expressed (Yamaguchi et al. 2000; Ripperger and Schibler 2006; Gachon 2007). Their protein products, in turn, act via D-box elements to regulate gene expression (Ueda et al. 2005). Among the genes regulated by these rhythmically expressed transcription factors are *Per1 and Per2*, thus forming another potential interlocked feedback loop (Yamaguchi et al. 2000; Mitsui et al. 2001; Ueda et al. 2005; Ohno et al. 2007), and indeed, DBP and E4BP4 are important for period determination (Yamajuku et al. 2011). In liver, the transcriptional targets of this gene family include genes involved in bile acid production (cholesterol 7-α hydrolase, Cyp7a in Fig. 1.5c) and in xenobiotic metabolism (Gachon et al. 2006; Gachon 2007; Noshiro et al. 2007). Circadian regulation of PAR bZIP genes is an important regulator of daily rhythms in drug responses.

Two bHLH-PAS proteins (DEC1 and DEC2) are also clock-controlled genes that influence the oscillatory mechanism. The DECs interfere with CLOCK:BMAL1 activity (Honma et al. 2002) by competing for E-box elements in responsive genes, and they also can interact with CLOCK:BMAL1 protein complexes to inhibit their activity (Honma et al. 2002; Li et al. 2004). Absence of DEC1 and DEC2 has modest effects on molecular and behavioral rhythmicity (Grechez-Cassiau et al. 2004; Rossner et al. 2008).

The product of a gene previously known as *Gene model 129* (*Gm129*) is important for the regulation of circadian rhythms. In recognition of its role as a circadian modifier, *Gm129* has been renamed "*Chrono*" (Anafi et al. 2014; Goriki et al. 2014). *Chrono* is expressed rhythmically, the protein binds to E-box elements rhythmically, and it represses CLOCK:BMAL1 activated gene expression (Anafi et al. 2014; Annayev et al. 2014; Goriki et al. 2014). CHRONO interacts with BMAL1 and also with the glucocorticoid receptor. These reports differ on whether CHRONO repressed CLOCK:BMAL1 activated gene expression by disrupting interaction with the coactivator protein CBP versus whether it altered interaction with histone deacetylase 1 (HDAC1). *Chrono* knockout mice and mice lacking CHRONO specifically in vasopressin neurons have a very small but statistically significant increase in circadian period in constant conditions (Anafi et al. 2014; Goriki et al. 2014), while a third study simply stated that their experiment was not powered adequately to detect small differences in free-running period (Annayev et al. 2014). Collectively, these studies show that *Chrono* is a clock-controlled gene product that forms another interlocking feedback loop.

1.5.6 Setting Clock Speed by Regulation of Repressor Complex Stability

A molecular feedback loop involving transcription and translation, followed by negative feedback on transcription, could easily be accomplished in a few hours. For example, a variety of stimuli induce c-*fos* gene expression in brain, with peak transcript levels occurring 20–30 min after the stimulus and with protein levels peaking at 1–2 h. Similarly, a biological clock regulating somites during development has a similar transcriptional–translational feedback loop structure as the circadian clock, and yet this cycle has a period length of 2 h (Hirata et al. 2002). To achieve the near-24-h cycle length of circadian rhythms must involve careful, and extensive, regulation of the molecular events in rhythmic cells. What are the regulatory events that slow the molecular feedback loop sufficiently to achieve a cycle length of 24 h?

As noted above, the negative feedback effectors for circadian rhythms are complexes of PER and CRY proteins. Phosphorylation, ubiquitination, and proteasomal degradation are key steps in the regulation of their half life, and manipulating these processes affects the speed of the circadian clock. For reviews of the role of these posttranslational mechanisms in regulating circadian rhythms, see Gallego and Virshup (2007) and Reischl and Kramer (2011).

The PER proteins are substrates for several protein kinases that lead to their phosphorylation, ubiquitination, and proteasomal degradation. The kinases involved in posttranslational regulation of PERs include casein kinase 1 (CK1) δ and CK1 ε. Disruption of CK1 δ has modest but significant effects on circadian rhythm period (e.g., a 1–2 h increase) in cellular or tissue assay systems (Etchegaray et al. 2009, 2010). In humans, a mutation of CK1 δ is one cause of familial advanced sleep phase syndrome (Xu et al. 2005). Behavioral rhythms cannot be examined in CK1 δ knockout mice because these animals die within hours of birth (Xu et al. 2005; Etchegaray et al. 2009). CK1 δ appears to play a more important role in period regulation than CK1 ε, and drugs that affect both isoforms have a more pronounced effect than genetic disruption of either isoform alone or selective inhibition of CK1 ε (Meng et al. 2008; Etchegaray et al. 2009, 2010; Walton et al. 2009). Drugs with less CK1 isoform specificity markedly increase circadian cycle length (Walton et al. 2009; Hirota et al. 2010), suggesting functional overlap between CK1 δ and CK1 ε (and perhaps other kinases). Small molecule screens also suggest that inhibition of several kinases at once produces a very potentiated response (Hirota et al. 2010). A spontaneous mutation of CK1 ε identified in Syrian hamsters (the *tau* mutation) leads to shortened period (Ralph et al. 1990; Lowrey et al. 2000). This mutation produces a similar semi-dominant, short-period phenotype when "knocked in" to CK1 ε in mice (Meng et al. 2008). The *tau* mutant CK1 ε has increased activity at phosphorylating PERs and promoting their degradation (Gallego et al. 2006a). The effects of the casein kinases on regulation of period length require PER proteins (Maywood et al. 2014). Two other

casein kinases, CK1 α and CK2, also have roles in regulating circadian cycle length (Maier et al. 2009; Tsuchiya et al. 2009; Hirota et al. 2010).

Phosphorylation of the PER proteins by casein kinases is opposed by protein phosphatases, especially protein phosphatase 1 (Gallego et al. 2006b; Lee et al. 2011; Schmutz et al. 2011) and protein phosphatase 5 (Partch et al. 2006) and this balance is essential for setting clock speed.

Phosphorylation precedes and enables ubiquitination by E3 ubiquitin ligases, promoting proteasomal degradation. Not surprisingly, several F-box proteins and ubiquitin–proteasome-related mechanism influence circadian clock function. The F-box proteins β-TrCP1 and B-TrCP2 are ubiquitin ligase, catalyzing addition of ubiquitin to substrates (including PER1 and PER2) and promoting their proteasomal degradation (Shirogane et al. 2005; Reischl et al. 2007; Ohsaki et al. 2008). PER degradation can be inhibited by disrupting B-TrCP or by inhibiting proteasomal degradation (e.g., with MG132), lengthening circadian period.

Two F-box proteins, FBXL3 and FBXL21, regulate the degradation of CRY proteins. Null mutations of *Fbxl3* increase circadian period length (Busino et al. 2007; Godinho et al. 2007; Siepka et al. 2007a), while null mutations in *Fbxl21* decrease circadian period (Dardente et al. 2008; Hirano et al. 2013; Yoo et al. 2013). These proteins compete with and oppose each other's actions (Dardente et al. 2008; Hirano et al. 2013; Yoo et al. 2013). While CRY1 and CRY2 are both inhibitors of CLOCK:BMAL1-mediated transcription, mice with disruption of these genes have opposite effects on period length in constant darkness (Vitaterna et al. 1999; Van der Horst et al. 1999). The balance between the two CRY proteins (with CRY1 having higher activity compared to CRY2 as a transcriptional repressor), as well as the balance between these two F-box proteins, plays a major role in setting clock speed (Anand et al. 2013; Hirano et al. 2013; Yoo et al. 2013). A small molecule screen for compounds that alter the period of circadian oscillations in vitro identified KL001, which was shown to interact with CRY proteins and slow their proteasomal degradation, leading to an increase in period length in cellular assays (Hirota et al. 2012).

While this discussion has focused on stability of the PER:CRY complex (the most highly dynamic, temporally regulated components of the circadian oscillator), other circadian clock proteins are substrates for these kinases as well. Secondary phosphorylation occurs in complexes containing kinase:PER:CRY, and phosphorylation can also occur directly when kinases are expressed with other circadian proteins in vitro. CLOCK undergoes rhythmic changes in its phosphorylation state (Lee et al. 2001), affecting its activity and stability.

Lithium, a mood stabilizer used to treat bipolar disorder in humans, has long been known to increase the period length of circadian rhythms in cells, tissues, and whole organisms. The cellular target of lithium has been thought to be glycogen synthase kinase 3 (GSK3), which is inhibited by lithium (Can et al. 2014). Inhibition of GSK-3 destabilizes REV-ERB-α, thus affecting BMAL1 expression and other components of the molecular clockwork (Iitaka et al. 2005). GSK3-β has also been proposed as a kinase involved in the phosphorylation of CLOCK (Spengler

et al. 2009) and BMAL1 (Sahar et al. 2010). While lithium inhibits GSK-3-β and increases circadian period, more selective inhibitors of GSK-3-β *decrease* circadian period (Hirota et al. 2008). Activation of GSK-3 *increases* circadian period (Paul et al. 2012). How increasing and decreasing GSK-3 activity could both lengthen the period is unclear and perhaps suggests that there are additional, clock-relevant targets for lithium (Can et al. 2014). Lithium also inhibits myo-inositol monophosphatase (IMPase), and mice with a point mutation in the corresponding gene (leading to loss of enzymatic activity) have a very small increase in circadian period length (Ohnishi et al. 2014), suggesting that this target alone is insufficient to explain the period-lengthening effects of lithium. See also Chap. 13 (Burris).

The product of the *Drosophila timeless* gene, dTIM, is a critical component of the fly molecular clock, serving with *Drosophila* dPER in the repressor complex in a manner similar to the role of CRY1 and CRY2 in mammals (Weaver and Emery 2013). Upon identification of an apparent TIM homolog in mammals (and prior to the recognition of the role of mammalian CRYs), mammalian TIM (mTIM) was presumed to be an essential binding partner for PERs and a component of the circadian clockwork in mammals. The role of mTIM in mammalian circadian timekeeping remains unclear, however (see Gotter 2006, for review). mTIM is more closely related to *Drosophila timeout* than to dTIM. While mTIM is abundant in complexes containing CRY proteins and can inhibit CLOCK:BMAL1-mediated transcriptional activation in mammalian cells, it cannot do so when expressed in S2 cells, suggesting that its interactions with the activator complex may be indirect. Mice lacking mTIM die before midgestation, precluding behavioral analysis. TIM has non-circadian roles related to chromosome structure (Gotter 2006). mTIM has been reported to affect circadian cycle length and to mediate the effect of DNA damage on circadian phase (Engelen et al. 2013). Previously, an isoform-specific role for mTIM in regulating SCN rhythmicity was proposed (Barnes et al. 2003). mTIM may be involved in circadian regulation by titrating CRY availability. Most recent reviews of the mammalian clockwork do not discuss mTIM, suggesting that, to most authors, its role remains unclear.

1.5.7 A Myriad of Mechanisms for Transcriptional Modulation

A number of accessory proteins, cofactors, and histone-modifying enzymes contribute to the molecular oscillator driving circadian rhythms. Many proteins interact with CLOCK and/or BMAL1 and influence their activity as transcriptional activators. The PER:CRY-containing complexes that mediate transcriptional repression do so by recruiting corepressors and/or antagonizing the activity of CLOCK:BMAL1-recruited transcriptional co-activators. This section will describe some of these interactions and the proteins that participate in them. For review, see Chong et al. (2012) and Partch et al. (2014).

1.5.7.1 Transcription Factors and Cofactors

A fruitful approach to identification of additional clock-regulatory components has been the isolation of proteins contained within complexes with other circadian clock proteins.

NONO (Non-POU domain containing, octamer-binding protein) was isolated as a component of the PER:CRY repressive complex (Brown et al. 2005). Subsequent work showed that all three members of the DBHS gene family (for *Drosophila* Behavior Human Splicing), NONO, SFPQ, and paraspeckle component 1 (PSPC1), play redundant roles in circadian regulation via E-box elements within the promoters of circadian genes (Guillaumond et al. 2011; Kowalska et al. 2012). NONO also couples the circadian clock to the cell cycle (Kowalska et al. 2013). CAVIN-3 is a PER2-interacting protein that influences circadian cycle length through regulation of PER:CRY interactions (Schneider et al. 2012).

BMAL1-containing complexes contain Receptor for Activated C Kinase-1 (RACK1) and protein kinase C-α (PKC-α) during the negative feedback phase of the cycle (Robles et al. 2010). These components suppress CLOCK:BMAL1 transcriptional activity, possibly through phosphorylation of BMAL1. Reducing RACK1 or PKC-α expression shortened circadian period (Robles et al. 2010; Nam et al. 2014). The chromatin remodeling enzyme LSD1 appears to be a key target of PKC-α. Thus, the PKC signaling pathway contributes to the circadian feedback loop by affecting circadian clock proteins as well as chromatin.

BMAL1-containing complexes also contain TRAP150 [thyroid hormone receptor-associated protein-150 (Lande-Diner et al. 2013)]. TRAP150 is rhythmically expressed and promotes CLOCK:BMAL1 binding to promoters of target genes, enhancing transcriptional activation. Depletion of TRAP150 blunts transcriptional activation and leads to low-amplitude, long-period rhythms (Lande-Diner et al. 2013).

Isolation of CLOCK-containing protein complexes leads to the identification of "CLOCK-Interacting Protein, Circadian" (CIPC) as an additional negative-feedback regulator of the circadian clock (Zhao et al. 2007). CIPC is rhythmically expressed and inhibits CLOCK:BMAL1 activity. Depletion of CIPC shortens the circadian period length.

Heat-shock factor 1 (HSF1) and Upstream Stimulating Factor 1 (USF1) have been identified as circadian transcription factors (Reinke et al. 2008; Buhr et al. 2010; Shimomura et al. 2013). Buhr et al. (2010) propose that glucocorticoids and temperature, which both impact the circadian clock in peripheral tissues, do so through modulation of HSF1 activity. USF1 expression differs in a strain-dependent manner and contributes to strain-dependent suppression of the long-period phenotype in $Clock^{\Delta 19/+}$ (Shimomura et al. 2013). The melatonin synthesis pathway also can suppress the phenotype of $Clock^{\Delta 19/+}$ (Shimomura et al. 2010), but apparently does so through effects on coupling of SCN oscillators rather than through a transcriptional mechanism.

Other recent papers describe roles for metastasis-associated protein 1 (Li et al. 2013a), the nuclear envelope protein MAN1 (Lin et al. 2014), and hepatic steroid receptor coactivator 2 (Stashi et al. 2014) as regulators of circadian rhythmicity, primarily through effects on the positive limb of the core feedback loop. CLOCK and BMAL1 are SUMOylated, and this posttranslational modification enhances the complex's transcriptional activity (Cardone et al. 2005; Lee et al. 2008; Li et al. 2013b).

1.5.8 Chromatin Remodeling and the Epigenetics of Circadian Timekeeping

In chromatin, DNA is wrapped around complexes of histone proteins in structures called nucleosomes. Posttranslational modifications of histones strongly influence the accessibility of the DNA to transcription factors. In this way, the "histone code" influences gene expression, which is often referred to as epigenetic regulation because the histones provide information to regulate gene expression that is above and beyond the elements found in the coding sequence of the DNA.

There are many posttranslational modifications of histones include phosphorylation, acetylation, methylation, ADP-ribosylation, and ubiquitination that can influence gene expression (for review, see Marmorstein and Trievel 2009). These site-specific modifications are mediated by specific enzymes, but also occur cooperatively (e.g., phosphorylation at one residue can increase the probability of acetylation at another residue). Several histone-modifying enzymes have been shown to influence circadian rhythms. For review of epigenetic influences on circadian clock function, see Ripperger and Merrow (2011), Feng and Lazar (2012), and Masri and Sassone-Corsi (2013).

1.5.8.1 Histone Phosphorylation

Light exposure that resets the phase of circadian rhythms globally increases histone H3 phosphorylation at Serine residue 10 in mouse SCN (Crosio et al. 2000).

1.5.8.2 Histone Acetylation and Deacetylation and the Link to Metabolism

Histone acetylation is regulated by the opposing activities of histone transferases (HATs) and histone deacetylases (HDACs). CLOCK has been reported to have HAT activity (Doi et al. 2006). Rhythmic histone H3 (but not H4) acetylation occurs in promoter-associated histones of rhythmically regulated genes (Etchegaray et al. 2003). The NAD(+)-dependent enzyme SIRT1 functions as a

histone deacetylase, counteracting the HAT activity of CLOCK (Asher et al. 2008; Nakahata et al. 2008). This provides a mechanism for cellular redox and metabolic state to influence circadian rhythmicity (see below). CLOCK also regulates transcriptional activity through acetylation of proteins: BMAL1 (Hirayama et al. 2007) and acetylation of the glucocorticoid receptor (Nader et al. 2009).

The histone acetyltransferase CBP/p300 is present in CLOCK-containing complexes from liver nuclear extracts, and the abundance of this complex at *Per* gene promoters is rhythmic (Etchegaray et al. 2003). CLOCK:BMAL1 transcriptional activity is enhanced by CBP/p300 and is reduced by CRY proteins. CRYs appears to neutralize the activity of CLOCK-containing complexes, reducing HAT activity, and altering chromatin structure in ways that blunt transcriptional activation, leading to negative feedback (Etchegaray et al. 2003). CBP has been proposed to play an important role in clock resetting (Lee et al. 2010). Hosoda et al. (2009) reported that p300's activity could either augment or repress CLOCK:BMAL1-mediated transcriptional activity in a cell-line-specific manner, which related to the abundance of the corepressor histone deacetylase 3 (HDAC3) and the coactivator pCAF in the cell lines.

Transcriptional repression by the PER complex involves assembly of a PER1:SFP:SIN3A complex, which recruits HDAC1, leading to deacetylation of H3K9 at CLOCK:BMAL1 target sites, including the *Per1* promoter (Duong et al. 2011).

The nuclear orphan receptor REV-ERB-α (NR1D1) is in fact no longer an "orphan," as its ligand has been identified: it is a receptor for heme (Yin et al. 2007). *Rev-erb-α* gene is rhythmically expressed in many tissues, where it directly represses expression of *Bmal1* (Kornmann et al. 2007). REV-ERB-α also regulates many metabolic genes. Mechanistically, REV-ERB-α represses transcription by recruitment of a Nuclear CoRepressor-HDAC3 complex, leading to rhythmic histone deacetylation and wide-ranging effects on gene expression (Alenghat et al. 2008; Feng et al. 2011). Disruption of both *Rev-erb-α* and *Rev-erb-β* disrupts rhythmic clock gene expression and circadian behavioral rhythms and also leads to deregulated lipid metabolism and hepatic steatosis (Yin et al. 2007; Bugge et al. 2012; Cho et al. 2012). Thus, the rhythmic expression of REV-ERB-α plays a key role in linking circadian rhythms with metabolism and occurs through rhythmic epigenetic regulation mediated by changes in histone proteins (Ripperger and Merrow 2011; Masri and Sassone-Corsi 2013).

Other metabolic influences on circadian clock function interface with histone modifications (for reviews, see Jordan and Lamia 2013; Masri and Sassone-Corsi 2013). The rate-limiting enzyme in mammalian NAD+ biosynthesis is nicotinamide phosphoribosyltransferase (NAMPT). *Nampt* gene expression, NAMPT activity, and levels of NAD+ are rhythmic (Nakahata et al. 2009; Ramsey et al. 2009). The *Nampt* gene is a direct transcriptional target of CLOCK:BMAL1 activity. SIRT1, a HDAC, opposes CLOCK:BMAL1 activity, while NAMPT inhibition releases this repression. Thus, redox state influences transcriptional activator activity and gene expression rhythms through histone modifications. In addition, the DNA binding of transcriptional activator complexes is regulated by redox state. More specifically, transcriptional activity of CLOCK:BMAL1 and NPAS2:BMAL1 complexes is

increased by the reduced state of the redox cofactors NAD(H) and NADP (H) (Rutter et al. 2001; Yoshii et al. 2013).

The activity of AMP-activated protein kinase (AMPK) oscillates in a circadian manner; AMPK phosphorylates CRY1, leading to its destabilization and to derepression of circadian transcription (Lamia et al. 2009). The importance of this is that cellular cAMP signaling status can directly influence circadian clock function, and in fact clock function is also dependent on cellular signaling mechanisms including cAMP and calcium (O'Neill et al. 2008; Atkinson et al. 2011; Noguchi et al. 2012; Hastings et al. 2014; Pauls et al. 2014). PGC1-α and PPAR-γ also provide molecular links between circadian clocks and metabolism (Liu et al. 2007b). A more extensive discussion of the interface between circadian clocks and metabolism appears in Chap. 5 by C. B. Green. (See also Peek et al. 2012; Jordan and Lamia 2013; Masri and Sassone-Corsi 2013.)

1.5.8.3 Histone Methylation

In the *Dbp* gene, circadian rhythms of H3K9 acetylation and dimethylation peak in opposite phases, with the peak in acetylation occurring coincident with transcription and the peak of dimethylation occurring during the repression phase of the circadian cycle (Ripperger and Schibler 2006).

Duong and Weitz (2014) recently reported that mouse PER protein complexes include a histone methyltransferase activity from the proteins HP1γ-Suv39h1-2 (suppressor of variegation 3–9 homolog 1 and 2). PERs recruit the HP1γ-Suv39h complex to the *Per1* and *Per2* promoters, where they contribute to the rhythmic di- and trimethylation of histone H3K9. Reducing expression of this complex leads to increased expression of CLOCK:BMAL1 targets (by derepression) and shortens circadian period. The alternation between histone acetylation and methylation, with active HDAC1-mediated deacetylation of H3K9, enabling subsequent methylation of H3K9, is important for feedback repression and circadian clock function (Ripperger and Schibler 2006; Duong and Weitz 2014).

Several other histone methyltransferases are important for circadian rhythms. Depending on the histone residues affected, these histone modifications can either repress or activate gene expression. EZH2 (enhancer of zeste homolog 2) coprecipitates with CLOCK:BMAL1 complexes and participates in CRY-mediated transcriptional repression (Etchegaray et al. 2006). EZH2 is a component of "polycomb repressive complex 2" and catalyzes the di- and trimethylation of lysine 27 on histone H3 (H3K27). Disruption of EZH2 expression leads to loss of cellular rhythms in vitro (Etchegaray et al. 2006). Mixed lineage leukemia 1 (MLL1) methylates lysine residue 4 (K4) on histone H3 and is also present in CLOCK:BMAL1 complexes. MLL1 leads to rhythms in this "activation mark" and H3K4 trimethylation appears permissive for rhythmic gene expression (Katada and Sassone-Corsi 2010). Valekunja et al. (2013) reported that rhythmic H3K4 trimethylation occurs at many loci, is required for rhythmicity of gene expression, is mediated by Mixed lineage leukemia 3 (MLL3), and this activity is independent

of MLL1, CLOCK, and BMAL1. Thus, multiple proteins and multiple molecular mechanisms act in concert to generate circadian rhythms in gene expression through histone methylation.

WDR5 was identified as a PER1-interacting protein, and it also binds to PER2 (Brown et al. 2005). WDR5 is part of a histone methyltransferase complex and is important for H3K4 trimethylation and H3K9 dimethylation at the Rev-erb-α promoter (Brown et al. 2005). Reducing WDR5 levels reduces histone methylation at PER1-regulated promoters, but does not alter circadian period length (Brown et al. 2005).

A histone lysine demethylase JARID1a (Jumonji, AT-rich interactive domain 1a, also called lysine (K)-specific demethylase 5A, or KDM5A) activates CLOCK–BMAL1 activity and influences circadian clock function (DiTacchio et al. 2011). Another Jumonj domain protein with histone demethylase activity, JMJD5 (also called KDM8), is important for circadian period regulation in both plants and mammalian cells. Knockdown of JMJD5 shortens circadian period (Jones et al. 2010).

Methylation of histone H4 arginine residue 3 (H4R3) by the type II protein arginine methyltransferase 5 (PRMT5) is a histone modification thought to be important for the regulation of *Per1* gene expression (Na et al. 2012). In mammalian cells, CRY1:PRMT5 interactions appear to promote histone H4R3 dimethylation, while reducing expression of PRMT5 reduces H4R3 dimethylation and influences *Per1* expression (Na et al. 2012).

1.5.8.4 Histone Substitution

Menet et al. (2014) recently reported that the CLOCK:BMAL1 complex promotes rhythmic chromatin opening in part through incorporation of a histone variant, H2A.Z, and this enables binding of other transcription factors adjacent to CLOCK:BMAL1 binding sites, increasing the diversity and complexity of circadian influence on gene expression profiles.

1.5.8.5 DNA Methylation

Methylation of DNA is distinct from methylation of histone proteins, but also can influence gene expression. Long-term housing of mice in non-24-h "T" cycles leads to "aftereffects" on the period length of locomotor activity rhythms. Azzi et al. (2014) recently showed that global changes in SCN DNA methylation result from exposure to these non-24-h lighting cycles. Both DNA methylation and behavioral effects were reversible with time and were prevented by infusion of a DNA methyltransferase inhibitor into the SCN. While the emphasis on histone posttranslational modifications is in orchestration of gene expression on a daily basis, these results show that long-term plasticity of the circadian clock can be achieved by long-term alterations in DNA methylation.

1.5.9 Post-transcriptional Regulation of the Circadian Clock

The previous sections emphasized how posttranslational modifications of core clock components and histone proteins can influence circadian clock function. In this section, I will review another level of post-transcriptional control, addressing regulation of transcript stability and competence for translation. The extent of the post-transcriptional regulation of gene expression is striking: recent studies reveal that many more transcripts are rhythmically expressed than can be accounted for based on rhythmic transcription (Rey et al. 2011; Koike et al. 2012; Menet et al. 2012; Kojima and Green 2015). Thus, many mRNA rhythms must result from post-transcriptional mechanisms.

1.5.9.1 MicroRNAs

One mechanism of epigenetic regulation of circadian clock function is through microRNA-mediated post-transcriptional gene silencing. MicroRNAs are products of noncoding genes that interact with transcripts from coding genes to inhibit their expression. Disruption of Dicer, an enzyme necessary for microRNA generation from precursor transcripts, affects the shape of gene expression profiles but does not lead to loss of rhythmicity (Chen et al. 2013; Du et al. 2014). Many regulatory RNAs (microRNAs and long noncoding RNAs) are rhythmically expressed and contribute to rhythmicity of protein-coding genes (Vollmers et al. 2012). Several specific examples of microRNA–circadian transcript interactions have been reported (Cheng and Obrietan 2007; Cheng et al. 2007; Gatfield et al. 2009; Alvarez-Saavedra et al. 2011; Shende et al. 2011, 2013; Lee et al. 2013b). Because of the sequence-specific nature of microRNA interactions with their mRNA targets, a huge number of combinations are possible. For relevant reviews, see Mehta and Cheng (2013), Kojima and Green (2015), and Shende et al. (2014).

1.5.9.2 Poly-A Tail Length

Variation in transcript poly-A tail length can influence transcript stability and the ability of the transcript to be translated. Circadian oscillations in poly-A tail length occur for some transcripts (Robinson et al. 1988; Kojima et al. 2012; Liu et al. 2013), leading to circadian rhythms in protein levels (Kojima et al. 2012). See Kojima and Green (2015) for further discussion.

1.5.9.3 Alternative Splicing and RNA-Binding Proteins

Immediately following transcription, pre-mRNAs are spliced, removing introns. Many genes can encode multiple, distinct protein isoforms as a result of differential

inclusion of exons from within the pre-mRNA. Even in the absence of differences within coding sequences, alternative splicing can influence gene expression levels because alternative promoter use and differences in 5′ untranslated regions can influence expression. Alternative splicing can also lead to a different 3′ untranslated region and thus different polyadenylation signals and differential inclusion of sites for microRNA interaction. Alternative splicing is controlled primarily through interactions of transcripts with RNA-binding proteins.

Several recent studies reveal important contributions of alternative splicing and RNA-binding protein interactions to circadian oscillation and output mechanisms. The cold-inducible RNA-binding protein, CIRP, is important for setting circadian clock speed through interaction with *Clock* mRNA and effects on alternative polyadenylation (Morf et al. 2012; Liu et al. 2013). LARK, an RNA-binding protein identified for its effects on circadian rhythms in *Drosophila* activates PER1 protein expression in mammals (Kojima et al. 2007). Nocturnin is an RNA deadenylase identified in *Xenopus* as a highly rhythmic gene expressed at high levels at night. Mammalian nocturnin regulates poly-A tail length of output genes without affecting the core clock (Kojima and Green 2015; Partch et al. 2014), but has important contributions to the regulation of metabolism (see Chap. 5).

Mouse PER-containing complexes also contain the RNA helicases DDX5 and DHX9, active RNA polymerase II (Pol II) large subunit, and SETX, a helicase that promotes transcriptional termination (Padmanabhan et al. 2012). Negative feedback by the PER-containing complexes inhibits SETX activity, retarding release of RNA polymerase II from Per and Cry pre-mRNAs present within the complexes. Thus, the PER-containing complex recruits proteins that interfere with transcriptional re-initiation.

RNA methylation performed by the RNA-binding protein Mettl3 influences transcript processing and inhibition of this enzyme lengthens circadian period (Fustin et al. 2013). In flies, products of the *twenty-four* (*tyf*) and *ataxin-2* genes regulate circadian cycle length by affecting PER protein translation (Lim et al. 2011; Lim and Allada 2013a; Zhang et al. 2013). Tyf has no mammalian homolog, but Ataxin-2 does.

1.6 Mutant Mouse Models: Past, Present, and Future

Mice with targeted disruption (presumed null alleles) of many of the genes involved in the core feedback loop have been generated. (See Table 1.1 for a list of circadian clock genes and their alternative names.) These mice have been extremely useful in defining the role of specific genes in the circadian mechanism (see also Yu and Weaver 2011). A general principle revealed by these studies is that there is often redundancy of function among members of a gene family, and so disruption of multiple family members is required to see the most robust circadian phenotype. Double-knockout mice with disruption of *Clock* plus *Npas2* (DeBruyne et al. 2007a), *Cry1* plus *Cry2* (van der Horst et al. 1999; Vitaterna et al. 1999), or

Per1 plus *Per2* (Bae et al. 2001; Zheng et al. 2001) are viable yet lack circadian rhythms, while single knockouts of these genes have less robust circadian phenotypes. *Bmal1* appears to be an exception to this redundancy-of-function rule, as mice with disruption of *Bmal1* alone are arrhythmic (Bunger et al. 2000). The closely related gene, *Bmal2* (*Mop9*), is a CLOCK:BMAL1 target, however, so *Bmal2* expression is greatly reduced in BMAL1-deficient mice (Shi et al. 2010). There is loss of expression of both genes when *Bmal1* alone is targeted. Indeed, when BMAL2 is overexpressed from a promoter not requiring CLOCK:BMAL1 for expression, BMAL2 can restore rhythmicity in BMAL1-deficient cells (Shi et al. 2010).

The gene redundancy that appears to be a common finding within "circadian clock gene families" has important implications for assessing gene function in circadian rhythms. Single knockouts may be relatively insensitive for detecting functional contribution of a gene. Mutagenesis with ethyl-nitroso urea (ENU) generated point mutations and so can generate either null (loss-of-function, equivalent to knockouts), dominant-negative, or gain-of-function alleles. These latter two classes of alleles frequently give more robust phenotypes than the corresponding null allele (see below), due to the ability of these mutant gene products to affect the activity of an entire gene family.

One advantage of the knockout approach is that spatial and/or temporal control of gene disruption can be achieved using conditional alleles (for a review, see Birky and Bray 2014). Several clock-relevant genes are available in versions where critical regions are flanked by loxP sites ("floxed") making them sensitive to disruption by Cre recombinase ("Cre"). A battery of transgenic lines expressing Cre recombinase in specific tissues and cell types exists, allowing tissue-specific disruption of gene function. Floxed alleles of *Bmal1* have been generated by the Weitz and Bradfield laboratories (Storch et al. 2007; Westgate et al. 2008). These conditional *Bmal1* lines have been used in publications examining the role of BMAL1 in vasculature (Westgate et al. 2008), retina (Storch et al. 2007), liver (Lamia et al. 2008), pancreas (Marcheva et al. 2010; Sadacca et al. 2011), kidney (Tokonami et al. 2014), adipocytes (Paschos et al. 2012), pituitary gonadotropes (Chu et al. 2013), ovary (Liu et al. 2014), and brain (Musiek et al. 2013). Floxed alleles of *Clock* have been generated (DeBruyne et al. 2006) and used in studies of liver (DeBruyne et al. 2014). A conditional allele of CK1δ and two conditional alleles of CK1ε have also been generated and studied (Meng et al. 2008; Etchegaray et al. 2009). In addition, transgenic lines in which CLOCK, CLOCK$^{\Delta 19}$, or BMAL1 expression can be controlled with doxycycline have been generated (Hong et al. 2007; McDearmon et al. 2006).

It is important to keep in mind that these "circadian clock genes" are not involved only in circadian rhythms. A phenotype observed in a circadian mutant mouse may be the result of the pleiotropic effects of the gene, rather than due to the impact of loss of circadian clock function per se (see Yu and Weaver 2011 for review).

Spontaneously arising mutant alleles of several circadian clock genes have been identified. These semidominant point mutations include mutations in the genes

encoding human PER2 (Toh et al. 2001), human CK1δ (Xu et al. 2005), and hamster CK1ε (Lowrey et al. 2000). In addition, two new mutant lines of hamsters with circadian period phenotypes have been described [Monecke et al. (2011), M. Menaker et al. unpublished (see abstract to R21 NS079986)]. The mutated genes remain to be identified.

Induced mutagenesis (caused by exposing male mice to ENU), followed by behavioral screening of offspring arising from the mutant gametes), has led to isolation of many mutations affecting circadian clock function (Bacon et al. 2004; Vitaterna et al. 2006).

The most well known of the mutants generated by mutagenesis is in a gene called "circadian locomotor output cycles kaput," a.k.a. *Clock* (Vitaterna et al. 1994). Positional cloning and transgenic rescue identified the affected gene and the mutation in it (Antoch et al. 1997; King et al. 1997a). The mutant allele has an intronic nucleotide substitution that leads to skipping of exon 19 during the splicing process (King et al. 1997b). The mutant product, $CLOCK^{\Delta 19}$, retains capacity to interact with BMAL1, and to bind to DNA, but lacks the transcriptional activator activity of wild-type CLOCK (Gekakis et al. 1998). Behavioral studies of the mutant allele in combination with genomic deletions containing *Clock* indicated that the $CLOCK^{\Delta 19}$ protein acts as a dominant negative, actively interfering with the function of the wild-type gene product in heterozygotes (King et al. 1997a). To emphasize, the $Clock^{\Delta 19}$ mutation is not a null allele. Studies with targeted disruption of *Clock* ("knockout" mice) reveal that mice lacking CLOCK protein maintain robust behavioral circadian rhythms, albeit with a slightly shorter circadian period length in DD and altered responsiveness to light (DeBruyne et al. 2006; Dallmann et al. 2011). A related transcription factor, NPAS2, can maintain behavioral rhythmicity in the absence of CLOCK (DeBruyne et al. 2007a).

In a screen for dominant mutations affecting circadian rhythms in offspring of mutagenized males, a null allele of *Clock* likely would have been missed (e.g., $Clock^{+/-}$ have no discernible phenotype). This is the very type of screen that detected the $Clock^{\Delta 19}$ mutation. Subsequent mutagenesis screens have been designed to pick up recessive mutations/null alleles (e.g., Siepka et al. 2007a), but even with this design it is not clear if the modest change in period length of $Clock^{-/-}$ mice in constant darkness would have been detected. Thus, ENU mutagenesis generated a novel, dominant-negative allele of *Clock* with a robust phenotype detectable in heterozygotes, identifying a key circadian gene whose importance in circadian rhythms may have been overlooked had a knockout strategy been used. This raises questions about the promise of large-scale knockout projects for identifying circadian-relevant genes, in view of the pattern noted above that there is frequently redundancy among gene family members. Hogenesch and colleagues have used a combination of criteria to identify putative circadian clock genes (Anafi et al. 2014), which may improve detection of genes with more subtle but still important contributions.

Mutagenesis continues to reveal novel information about the molecular mechanisms of circadian clock function. Vitaterna et al. (2006) indicate they had recovered 46 confirmed mutants affecting circadian rhythms in the ENU-mutagenesis

screen performed at Northwestern University, as of December 2005. Of these, *Overtime* has been identified as a mutation in *Fbxl3* (Siepka et al. 2007a), *part-time* has been identified as a null allele of *Cry1* (Siepka et al. 2007b), and *Past-time* has been identified as a mutation in *Fbxl21* (Yoo et al. 2013). The molecular identity of the other mutants mentioned in this article (with names like *Half-time*, *Time course*, *Time machine*, *Time share*, *Time Trial*, *Time Traveler*) has not yet been reported. Additional mutants have been generated in a similar screen performed at Harwell, UK (Bacon et al. 2004). The Harwell screen led to the identification of the *Afterhours* mutation as an additional allele of *Fbxl3* (Godinho et al. 2007) and identification of the transcription factor zinc finger homeobox 3 (*Zfhx3*) as the gene mutated in the *Short-circuit* (*Sci*) mutant line, as discussed earlier (Parsons et al. 2014). Whether the other mutants identified in these screens represent new mutations in known clock genes, or new clock genes, remains to be determined. This abundant pipeline of yet-to-be-identified mutants affecting circadian rhythms, plus the continuing identification of novel circadian regulatory mechanisms (most recently, LHX1, ZFHX1, and CHRONO), indicates that there is potential for significant, continuing growth in our understanding of circadian clock mechanisms.

1.7 Some Inconvenient Truths

The sections above described the preeminent role of the SCN as the master circadian oscillator and defined the transcriptional/translational feedback loop as the molecular basis for rhythmicity. It is worth noting that there are some well-established facts that cannot be readily reconciled with the standard, semi-dogmatic views expressed above. The inconvenient truths are that (1) the SCN are not absolutely necessary for generation of circadian rhythms in locomotor activity, (2) the transcriptional–translational feedback loop highlighted above is not necessary for behavioral or molecular rhythms with a cycle length of ~24 h, and (3) sometimes the proteins of the feedback loop do not operate as predictably as their roles described above would indicate.

1.7.1 Circadian Rhythms in Locomotor Activity Can Exist in the Absence of a Functioning SCN

Numerous findings indicate the existence of one or more extra-SCN oscillator capable of engaging output pathways leading to rhythmic locomotor activity in rats and mice.

1.7.1.1 The Food-Entrainable Oscillator

Studies dating from the 1970s, many conducted by F. K. Stephan at Florida State University, demonstrated the existence of an SCN-independent food-entrainable oscillator (FEO) (Krieger et al. 1977; Stephan et al. 1979; for reviews, see Honma and Honma 2009; Mistlberger 2011; Carneiro and Araujo 2012). Restricting food availability to several hours per day for a week or more leads to the appearance of increased locomotor activity in anticipation of the daily time of food availability, even in SCN-lesioned rodents. An hourglass mechanism cannot explain this rhythmicity, as rhythmic activity recurs even during fasting and is masked, but not eliminated, by ad lib feeding. Food removal after even a week of ad lib feeding leads to restoration of the anticipatory activity appropriately timed to the previous period of restricted food availability. Heavy water, which increases the period of cellular and behavioral rhythms across phyla, does not affect the period of the FEO (Mistlberger et al. 2001). Molecular studies in SCN-intact rodents show that restricted feeding entrains clock gene rhythms in peripheral tissues but does not affect the SCN (Damiola et al. 2000; Stokkan et al. 2001). Other studies indicate a complex interaction between the FEO and the light-entrainable oscillator in the SCN (Mistlberger 2011; Pendergast et al. 2012; Pendergast and Yamazaki 2014).

The impact of mutations in circadian clock genes on the FEO is also unexpected. In homozygous $Clock^{\Delta 19/\Delta 19}$ mutant mice, transient circadian rhythms occur after release from a light:dark cycle, with a circadian period of ~28 h (Vitaterna et al. 1994). During exposure of these *Clock* homozygous mutant mice to a restricted feeding cycle, the FEO drives anticipatory activity with a cycle length of ~24 h, even in DD (Pitts et al. 2003). These results suggest that the molecular mechanism regulating circadian food anticipatory activity is distinct from the light-entrainable oscillator in the SCN. The effects of disruption of genes critical for the light-entrainable oscillator in the SCN on the food-entrainable oscillator appear variable among studies. Several studies report food anticipatory activity (FAA) with ~24-h rhythmicity in mice lacking functional alleles of *Per1*, *Per2* or both *Per1* and *Per2* (Storch and Weitz 2009) and in mice lacking BMAL1 (Mistlberger et al. 2008; Pendergast et al. 2009; Storch and Weitz 2009). Other studies report deficits in food entrainment in BMAL1-deficient mice (Mieda and Sakurai 2011; Takasu et al. 2012), in CRY1/CRY2-deficient mice (Takasu et al. 2012), and in *Per2*-mutant mice (but not in PER1-deficient mice) (Feillet et al. 2006). One controversial paper claims that $Bmal1^{-/-}$ mice lack food entrainment and that rescue of BMAL1 expression in the dorsomedial nucleus of the hypothalamus rescues food entrainment (Fuller et al. 2008, 2009). Others dispute the role of BMAL1 and the DMH in food entrainment (Mistlberger et al. 2008, 2009a, b; Storch and Weitz 2009). Strong evidence for a functional food-entrainable oscillator in at least some studies of mutant mice and the clear presence of FAA in animals with complete SCN lesions indicates the presence of an SCN-independent oscillator that can serve as a pacemaker regulating locomotor activity rhythms.

1.7.1.2 The Methamphetamine-Sensitive Circadian Oscillator

Another SCN-independent oscillator that shares many properties with the FEO is the methamphetamine-sensitive circadian oscillator (MASCO), initially identified by Sato and Ken-Ichi Honma and colleagues (Honma et al. 1987). Allowing SCN-lesioned, arrhythmic rats to drink water containing a low concentration of methamphetamine led to restoration of robust locomotor activity rhythms with a period length of ~26 h. To rule out the possibility that this rhythmic behavior was due to rhythmicity in drug intake, additional studies were conducted in which methamphetamine was administered by mini-osmotic pumps, with similar results (Honma et al. 1987). More recent studies have shown that the MASCO exists in mice, with methamphetamine inducing rhythms with a period length ~26 h in SCN-lesioned C57BL/6J mice (Tataroglu et al. 2006). Methamphetamine-induced rhythms are observed even in mutant mice that lack SCN-driven circadian rhythmicity (*Bmal1*, *Per1*/*Per2*, and *Cry1*/*Cry2*; Honma et al. 2008; Mohawk et al. 2009). The MASCO is detectable in these mutant lines both with and without the destruction of the SCN (Mohawk et al. 2009; Pendergast et al. 2012, 2013; Pendergast and Yamazaki 2014), but destruction of the SCN has a substantial consequence on the period of rhythmicity even in genetically clock-deficient mice. These results indicate a complex interaction between the SCN and the MASCO (Pendergast et al. 2013).

1.7.1.3 Do the MASCO and the FEO Reflect the Same Mechanism?

The MASCO and FEO have generally similar properties, and it is widely speculated that they may share anatomical and/or molecular mechanisms leading to rhythmicity (Honma and Honma 2009). In fact, in mice lacking functional copies of all three *Per* genes, the FEO and MASCO both generate rhythms with a very short (~21 h) period (Pendergast et al. 2012). At present, the molecules and anatomical sites underlying the FEO and the MASCO remain unclear. What is clear, however, is that these oscillators, capable of acting as pacemakers regulating locomotor activity rhythms on a circadian timescale, do not reside in the SCN.

1.7.2 Circadian Biochemical Oscillations Exist in the Absence of Transcription

In cyanobacteria, the KaiA, KaiB, and KaiC genes are components of a ~24 h rhythmic molecular feedback loop (Johnson and Egli 2014). Remarkably, in vitro expression of these three proteins, in the absence of transcription, produced circadian rhythms in KaiC phosphorylation (Nakajima et al. 2005).

The capacity for biochemical oscillations with a cycle length of ~24 h in the absence of transcription and translation also exists in mammalian cells. This is obviously difficult to reconcile with the basis for the core circadian loop being a transcriptional/translational feedback loop. O'Neill and colleagues identified circadian rhythms in peroxiredoxins, with oscillation between reduced and oxidized states in vitro in the absence of nuclei and in the absence of transcription (O'Neill and Reddy 2011; O'Neill et al. 2011). The oscillation in peroxiredoxin oxidation state is observed across a wide representation of species (Edgar et al. 2012), fueling interest in this oscillation as a primitive metabolic oscillator that may have preceded the development of transcriptional/translational feedback loop mechanisms for circadian rhythms in cyanobacteria, fungi, plants, and animals (O'Neill et al. 2011). A question remaining is whether, and how, this transcription-independent molecular oscillation engages the transcriptional machinery to more widely affect cellular processes.

1.7.3 Circadian Clock Proteins Sometimes Act in Unpredictable Ways

In the canonical model of the transcriptional/translational feedback loop, PERs and CRYs are the repressor proteins. This role is well established in the SCN and in many cell lines. Studies by Gumz, Richards, and colleagues reveal that PER1 has an unexpected role, serving to increase expression of renal genes that are also the target of CLOCK:BMAL1 (Richards et al. 2013; see Chap. 6). This work suggests that PER1's role is to antagonize CRY2, removing the repressive effect of the CRY and so acting as an activator by a derepression mechanism, but this effect appears to be tissue and gene dependent. Nevertheless, activation of gene expression by a component of the repressor complex is clearly contrary to the canonical model in which PERs are key, rate-limiting factors for bringing CRYs to the nucleus to effect repression. The unique roles of circadian clock genes in individual tissues may be related to tissue-specific differences in gene-expression levels or of post-transcriptional regulatory mechanisms. See Chap. 6 for further discussion.

1.8 Summary and Prospectus

The intracellular mechanism for circadian rhythmicity is a transcriptional–translational feedback loop. This feedback loop is operative in many cell types and provides local regulation of rhythmic function. Rhythmic transcription is one component, but rhythmicity in metabolites, chromatin remodeling, and post-transcriptional regulatory mechanisms achieve rhythmicity with 24-h periodicity. Despite the cell-autonomous nature of circadian oscillators, intercellular

communication (including via neuropeptides) plays a key role in coordinating activity among oscillators within the SCN, binding them into a robust, functional pacemaker capable of regulating physiology and behavior. Oscillatory mechanisms independent of this mechanism exist, however. There is also a long list of ENU-induced mutations in mice that cause defects in circadian rhythmicity. These findings indicate that additional discoveries remain to be made into the mechanisms of rhythmicity. The following chapters describe the impact of circadian clocks in regulation of tissue-specific physiological processes.

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Chapter 2
The Circadian Timing System and Endocrine Physiology

Michael T. Sellix

Abstract From the pineal gland to the endocrine pancreas, the circadian timing system drives rhythms of hormone synthesis and secretion. Observation of rhythmic hormone secretion stimulated discovery in the field of chronobiology for well over a century and continues to present new and exciting challenges. Much of what we know about the biological basis of timing in physiology is fruit born of an effort to characterize the basis of hormonal oscillations. The advent of applied molecular genetics in circadian biology ushered in a wave of discovery, leading to characterization of the molecular basis of circadian rhythms in mammals. With this discovery came the revelation that nearly every cell and tissue in mammals harbors a circadian clock: a self-regulating transcription based oscillator of genetic regulatory factors. This benchmark stimulated an explosion of interest in the role for the molecular clock in the timing and amplitude of cellular and organismal physiology. As a field, interest rapidly turned to the elegant and persistent rhythms of hormone secretion. With this renewed focus, the last 10–15 years have been a time of high-paced discovery. We now know a great deal about molecular clock function in each of the major endocrine organs, including the pituitary gland, adrenal gland, pineal gland, gonads, adipose tissue, and endocrine pancreas. The impact of altered clock function in these tissues on physiology suggests strong functional links between the timing and endocrine systems. Understanding the depth and breadth of this integration will allow us to appreciate the complex mechanisms of basic endocrine physiology and possibly provide new and exciting avenues for the treatment of devastating and complex endocrine disorders.

Keywords Rhythm • Clock genes • Hormones • Metabolism • Homeostasis • Mammal

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Abbreviations

AANAT	Arylalkylamine *N*-acetyltransferase
ACTH	Adrenocorticotrophic hormone
AVP	Arginine vasopressin
BMAL1	Brain and muscle arnt-like protein 1
CCGs	Clock-controlled genes
CLOCK	Circadian like oscillatory cycles kaput
CORT	Corticosterone
CRH	Corticotropin-releasing hormone
CRY	Cryptochrome
COX2	Cyclooxygenase 2 (prostaglandin synthase)
DA	Dopamine
DBP	d-element binding protein
DD	Constant dark
EGR-1	Early growth response protein 1
FFA	Free fatty acids
FSH	Follicle-stimulating hormone
GC	Glucocorticoid
GnRH	Gonadotropin-releasing hormone
GnRH-R	Gonadotropin-releasing hormone receptor
HPA	Hypothalamic–pituitary–adrenal axis
HPG	Hypothalamic–pituitary–gonadal axis
L:D	Light:dark cycle
LH	Luteinizing hormone
LL	Constant light
LRH-1	Liver receptor homolog 1
MEL	Melatonin
NDNs	Neuroendocrine dopaminergic neurons
OVX	Ovariectomy
PAI-1	Plasminogen activator inhibitor 1
PTH	Parathyroid hormone
PTHr	Parathyroid hormone receptor
PER	Period
PGE2	Prostaglandin E2
PHDA	Periventricular hypophyseal dopaminergic neurons
PRL	Prolactin
PVN	Paraventricular nucleus
REV-ERBα	Reverse ERB alpha
RORα	Retinoic acid-like orphan receptor alpha
siRNA	Small interfering RNA
THDA	Tuberohypophyseal dopaminergic neurons
TG	Triglycerides
TIDA	Tuberoinfundibular dopaminergic neurons

T2D	Type-2 diabetes mellitus
VIP	Vasoactive intestinal polypeptide
WAT	White adipose tissue
WT	Wild type
3β-HSD	3-beta hydroxysteroid dehydrogenase

2.1 Biological Clocks in Endocrine Physiology: Rhythms of Life

Biological oscillations with a near 24-h period (circadian from the Latin *circa diem* "about one day") are the product of selective pressure of life on Earth. The precision of our planet's rotation on its axis and the persistence of daily solar illumination provided the framework for evolution of an intrinsic daily timing system. This internal timing system provides organisms with an evolutionary advantage: the ability to anticipate and adapt to predictable changes in the environment (both daily and yearly; Pittendrigh 1993; Moore-Ede et al. 1976). Moreover, it temporally compartmentalizes divergent physiological processes (e.g., lipolysis vs. esterification; bone ossification vs. bone resorption, etc.), thus maximizing efficiency and minimizing wasted energy. While the system shows great phylogenetic variability with regard to specific mechanisms, the overall goal is the same: to regulate with precision the timing of physiological function in order to maximize survival. This concept rings true from single cell cyanobacteria to plants, flies, and mammals (Gonze et al. 2002; Harmer et al. 2001; Panda et al. 2002, 2003). Precise temporal order is most apparent in the dynamic patterns of internal secretion. These are the rhythms of life: secretion of pituitary prolactin during the reproductive cycle, pregnancy, and lactation; rhythms of adipokine and insulin secretion from adipose tissue and pancreatic β cells, so critical for metabolic homeostasis; and the daily rhythms of gonadotropin secretion required in reproductive physiology. From birth, hormonal rhythms play a major role in defining the temporal order of our biological function, allowing us to develop, survive, and eventually pass on our genetic material. A clear understanding of the role of the biological or circadian timing system in mammalian endocrine physiology gives us a better understanding of our own chronophysiology, provides new insight into disease, and sheds ever-increasing light on the ties that bind us as passengers on Earth's 24 h journey. The goals of this discussion will be to (a) provide a basic understanding of the circadian timing system, from gene to physiology; (b) review how the timing system regulates hormone secretion in the major endocrine organs; and (c) explore the impact of clock disruption on homeostasis and pathophysiology.

2.2 The Circadian Timing System in Mammals

The circadian timing system regulates 24 h rhythms of physiology, gene expression, and behavior (Albrecht and Eichele 2003; Dibner et al. 2010; Albrecht 2012). As described in Chap. 1, biological rhythms are maintained at the molecular level by the cyclical transcription of circadian clock genes (see Fig. 2.1). Most, if not all, mammalian cells are host to an autoregulatory transcriptional–translational feedback loop of clock gene transcription factors (Mohawk et al. 2012; Albrecht 2012). The primary or core oscillator depends on the activity of the activators BMAL1 and CLOCK. The CLOCK protein is a histone acetyl transferase whose activity at target promoters depends on binding with its heterodimeric partner BMAL1 (Etchegaray

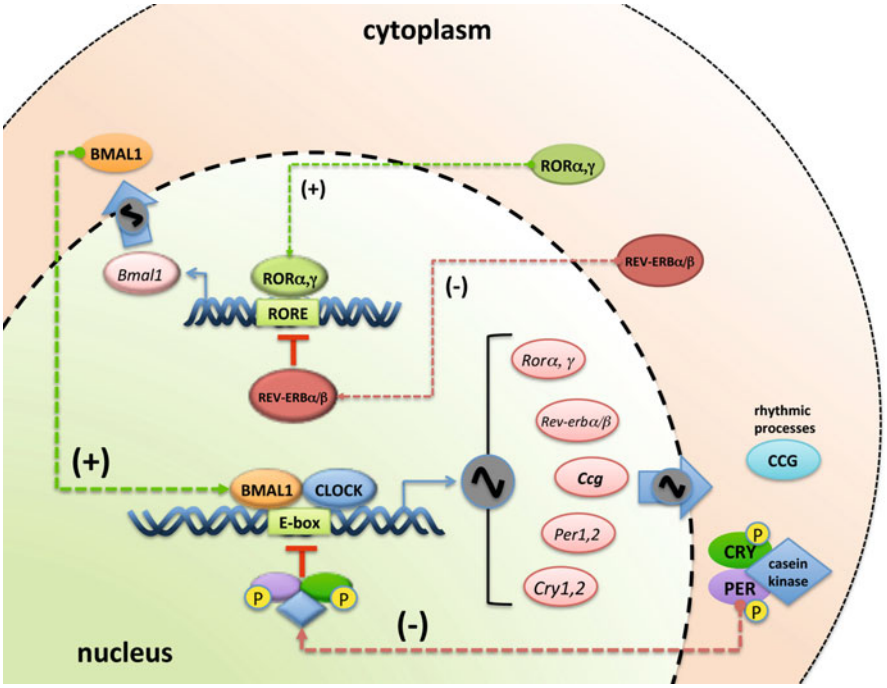

Fig. 2.1 The circadian oscillator in mammals. The mammalian circadian clock consists of a transcription based autoregulatory feedback loop. The positive arms of the loop include the transcriptional enhancers BMAL1 and CLOCK. Together, the BMAL1:CLOCK heterodimer binds to E-box sequences and enhances transcription of period (*per 1,2*), cryptochrome (*cry 1,2*), *reverbα, rorα*, and various clock-controlled genes (*ccgs*). PER and CRY proteins are post-translationally modified (e.g., phosphorylation) by casein kinases and translocate back to the nucleus where they repress BMAL1:CLOCK-dependent transcription. An additional loop provides stability and regulates the timing of *bmal1* expression. The heme receptor REV-ERBα also translocates back to the nucleus and suppresses the RORα-induced expression of BMAL1. The CCGs are the effector molecules of the oscillator, driving rhythmic processes in the cell ranging from hormone biosynthesis and secretion to glucose metabolism. *Abbreviations*: clock-controlled gene (*ccg*), phosphate (*P*)

et al. 2003; Hirayama et al. 2007; Nader et al. 2009). Together, the BMAL1:CLOCK complex binds to E-box (CACGTG) sequences in target promoters and drives transcription of the period (*per 1–3*) and cryptochrome genes (*cry1* and *cry2*). Following some critical posttranslational modifications, including phosphorylation by Casein Kinase, the PER and CRY proteins translocate back to the nucleus where they actively repress the activity of the BMAL1:CLOCK complex and suppress their own transcription. The delay in nuclear translocation of the repressor complex, controlled by parallel phosphorylation and protein degradation pathways, facilitates the roughly 24 h timing of the molecular oscillations. In addition to the core loop, a secondary loop of interlocking negative and positive transcriptional regulators including the repressor REV-ERBα and the enhancer retinoic acid-like orphan receptor α(RORα) provides stability and precision to the molecular timing system by regulating the timing of BMAL1 expression. Clock genes also directly regulate the timing of output genes or clock-controlled genes (CCGs). CCGs are the effector molecules of the clock, mediating its temporal control over physiological function (Kennaway et al. 2003; Hamada et al. 2004).

In mammals, the timing system has been described as a multi-oscillator hierarchy of coordinated and synchronized cell and tissue clocks (Fig. 2.2; Menaker et al. 2013; Davidson et al. 2003). At the top of this hierarchy is the central pacemaker located in the suprachiasmatic nucleus (SCN) of the basal hypothalamus. In addition to maintaining pacemaker function in the SCN, the molecular clock also regulates the timing of gene expression and physiology in peripheral tissues and extra-SCN brain regions (Yamazaki et al. 2000; Abe et al. 2002; Yoo et al. 2004; Menaker et al. 2013). It is now widely believed that, with minimal exceptions, the majority of cells in mammals are autonomous or semiautonomous circadian oscillators. This arrangement, as one can imagine, offers a unique set of challenges for the organism. How does the system effectively coordinate the timing of this panoply of clocks? What are the most efficient means to achieve this goal? Clearly, synchronization among central and peripheral oscillators (e.g., SCN, liver, pituitary gland, etc.) is a fundamental property of physiological homeostasis and a defining feature of the timing system (Fig. 2.3; Mohawk et al. 2012; Menaker et al. 2013; Guo et al. 2005; Buijs et al. 2001). This task largely falls to the coordinated timing of neural efflux from the SCN carried directly through peripheral nerves and more indirectly through patterns of hormone secretion driven by neuroendocrine and endocrine tissues (Fig. 2.2; Menaker et al. 2013; Albrecht 2012). Internal circadian organization is broadly defined as the coordinated and synchronized timing of central and peripheral clocks as well as the timing of each tissue clock relative to the external (and internal) environment (Fig. 2.3). A decline in circadian organization, referred to as circadian misalignment or chronodisruption (as seen during chronic jet lag, rotating shift work, etc.), can lead to numerous health issues including prediabetes, cancer, and cardiovascular disease (Litinski et al. 2009; Ruger and Scheer 2009; Scheer et al. 2009).

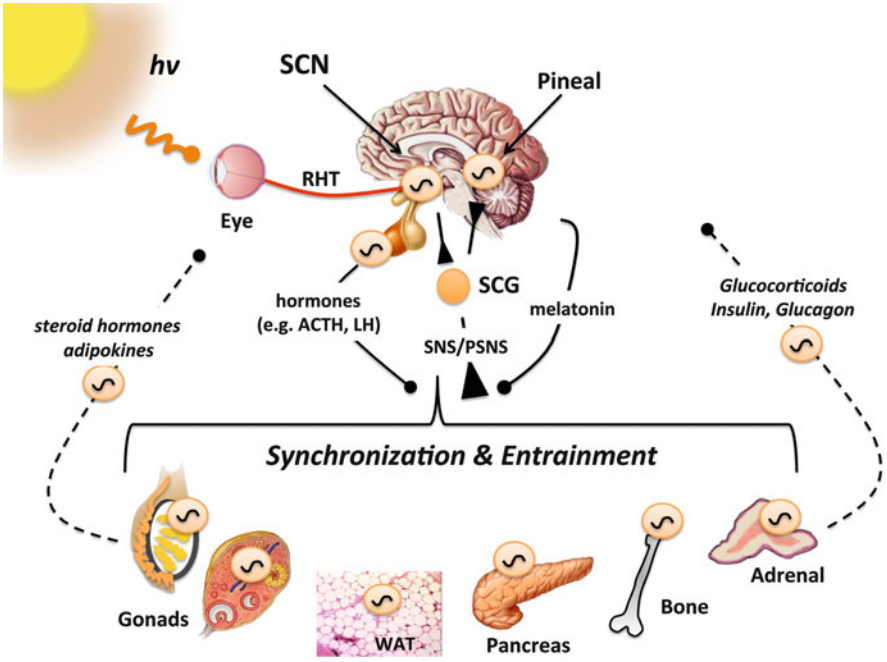

Fig. 2.2 Pathways of synchrony and entrainment in the endocrine system. A schematic of entrainment and synchronization pathways utilized to coordinate the oscillators of the endocrine system. Photic or time-of-day cues are perceived by the retina and transmitted to the central pacemaker in the SCN via the retinohypothalamic tract. These cues entrain the SCN to the external environment. The SCN then disperses this information to the remainder of the body via endocrine and neural signaling. The SCN communicates via the hypothalamus and ascending sympathetic nerves from the superior cervical ganglion to regulate the timing of pineal melatonin. Further, the SCN dictates the activity of neuroendocrine cells that in turn regulate patterns of pituitary hormone secretion. Finally, the SCN communicates entrainment cues to the periphery via descending sympathetic and parasympathetic nerves. These outputs (melatonin, SNS activity, etc.) converge on the major endocrine organs and regulate the timing of peripheral hormone secretion, cellular physiology, and gene expression. Further, the clock in each of the major endocrine organs contributes by affecting sensitivity to these central cues and driving cell-specific functions related to hormone secretion. Feedback from the major peripheral hormones, including the adipokines, glucocorticoids, insulin, and steroid hormones, can alter the timing of the clock in both a paracrine and endocrine manner. *Abbreviations*: light (hv), adrenocorticotrophic hormone (*ACTH*), luteinizing hormone (*LH*), suprachiasmatic nucleus (*SCN*), superior cervical ganglion (*SCG*), sympathetic nervous system (*SNS*), parasympathetic nervous system (*PSNS*)

2.3 The Circadian Timing System in Endocrine Physiology: From Pineal to Pancreas

The earliest evidence for clock function in the endocrine system was collected well before the SCN was identified as the neural locus of the mammalian pacemaker (Moore and Eichler 1972; Stephan and Zucker 1972). In the 1940s, Everett and

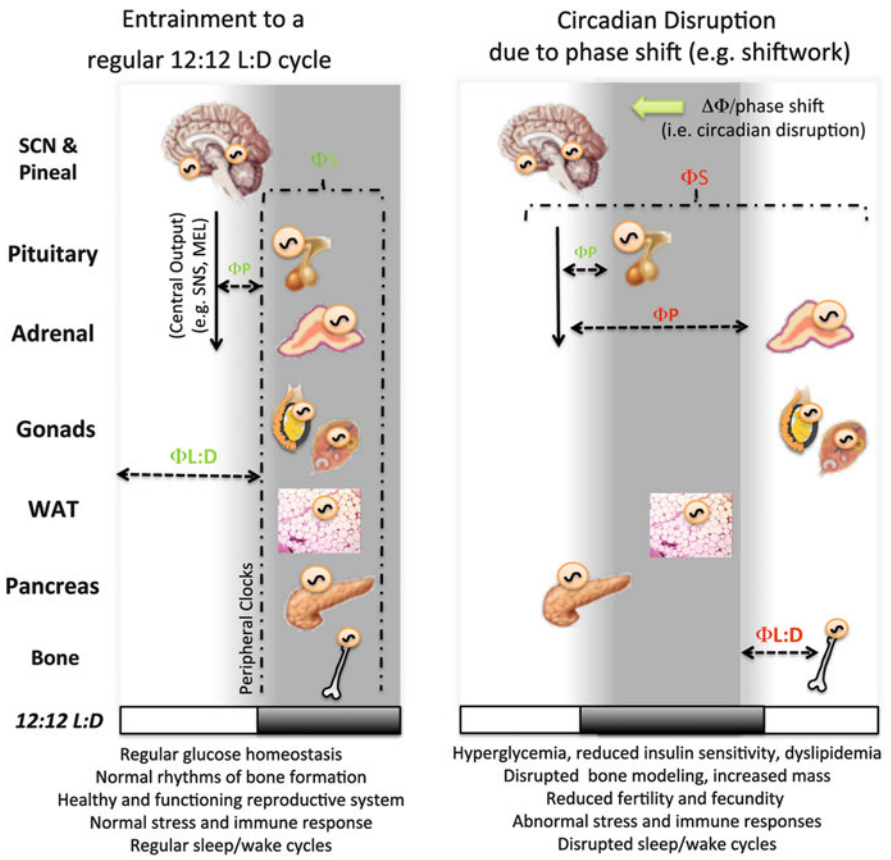

Fig. 2.3 Impact of circadian disruption on endocrine physiology. Acute or chronic misalignment between central and peripheral oscillators can have significant negative effects on regular endocrine physiology. When entrained to a normal 12:12L:D cycle, the circadian timing system is tightly coordinated and synchronized (*left panel*). The acrophase of gene expression and cellular physiology (indicated by the cartoon of each oscillator) is coordinated among the various tissues, coincident with normal daily rhythms of hormone secretion, glucose metabolism, bone modeling, and gonadal function. The phase relationship among the peripheral oscillators (phase spread; ϕS) is tight and well coordinated with the timing of sleep/wake patterns and feeding (limited to the night in rodents). Further, the phase relationship between the SCN and peripheral clocks (ϕP) is stable and consistent, as is the phase relationship between the L:D cycle and the oscillators (ϕL:D). Following acute or chronic phase shifts of the external environment or disruption of clock function due to genetic mutations (*right panel*), each of the phase markers defined above is disturbed. Particularly disruptive is the phase dissociation between the SCN and the majority of peripheral oscillators. The intensity and duration of this disturbance varies as a function of each oscillator. Regardless, the results are consistent; circadian misalignment alters glucose homeostasis, reproductive function, bone modeling, stress/immune responses, and sleep/wake cycles. *Abbreviations*: suprachiasmatic nucleus (*SCN*), melatonin (*MEL*), light:dark cycle (*L:D*), phase (ϕ), phase shift ($\Delta\phi$), white adipose tissue (*WAT*), phase spread (ϕS)

Sawyer established the role of a neural oscillator or "timer" in the temporal control of the ovulatory surge of luteinizing hormone (LH) secretion. The authors found that blocking the LH surge with a timed injection of anesthetic in the early afternoon on the day of ovulation, a treatment that globally silenced neuronal activity, prevented the rise in LH (and thus ovulation) and delayed it exactly 24 h (Sawyer et al. 1949; Everett and Sawyer 1950). This experiment provided a key piece of data supporting the relationship between the central biological timing system and the endocrine system. The role of the SCN in the timing of endocrine physiology is now well established (Kalsbeek and Fliers 2013; Hastings et al. 2007; Guo et al. 2005). The SCN regulates endocrine physiology, from pineal to pancreas, via a complex interplay of neuroendocrine, endocrine, and autonomic nervous timing cues (Fig. 2.2; Guo et al. 2005; Hastings et al. 2007; Buijs et al. 2003a, b; Kriegsfeld and Silver 2006). Outside the brain, the molecular clock has been localized to each endocrine tissue, but our understanding of the functional significance of the clock in each hormone regulatory system is limited. It has long been suspected that tissue autonomous clock function contributes to the timing of hormone synthesis and secretion (Andrews and Folk 1964; Andrews 1971; Ungar and Halberg 1962). Since the cloning of the first mammalian circadian clock gene in the mid-1990s (King et al. 1997) and the determination that clock genes are global regulators of cell function (Albrecht 2012), there has been a considerable increase in the evidence linking the molecular clock to endocrine physiology (Sellix and Menaker 2011; Tonsfeldt and Chappell 2012; Williams and Kriegsfeld 2012; Kalsbeek et al. 2012; Prasai et al. 2011; Bass and Takahashi 2010; Huang et al. 2011; Sellix 2014). These discoveries have paved the way for new insight into the impact of chronodisruption in all its forms on endocrine physiology ranging from the treatment of diabetes and obesity to addressing the ever-growing rate of global infertility (Mahoney 2010; Bass and Takahashi 2010). By more closely exploring the activity of the clock and its target genes in endocrine tissues, novel functional relationships may emerge, stimulating insight into the connection between clock function and endocrine physiology. Our continued appreciation of the ramifications of circadian disruption on disease may enhance our ability to exploit targeted manipulation of the clock mechanism, thus providing new and exciting avenues for the treatment of endocrine disorders.

2.4 Circadian Clock Function in the Hypothalamo-Pituitary-Adrenal (HPA) Axis

Circulating glucocorticoid (GC; cortisol in humans, corticosterone (CORT) in rodents) levels display a daily oscillation that peaks at or near the onset of activity in mammals (Halberg et al. 1959a, b; Czeisler and Klerman 1999). The circadian rhythm of adrenal GC secretion may be the most dynamic and profound of the endocrine rhythms (Kalsbeek et al. 1998, 2011b, 2012; Jones et al. 1987; Bagdy

et al. 1991; Buijs et al. 1993, 1999; Fuchs et al. 1996; Atkinson and Waddell 1997). Disruption of this rhythm following lesion of the SCN was critical evidence used in defining the neural locus of the central pacemaker (Moore and Eichler 1972). Moreover, the measurement of GC secretion and rhythmic responsiveness to ACTH in vitro was an early indicator that circadian clock function could persist in isolated endocrine tissues (Andrews and Folk 1964; Ungar and Halberg 1962). Analyses of the pathways regulating GC secretion have provided significant insight into the mechanisms of peripheral entrainment by the SCN (Kalsbeek et al. 2012; Pezuk et al. 2012; Kiessling et al. 2010). Adrenal GC secretion is driven by rhythmic secretion of adrenocorticotropic hormone (ACTH) of pituitary origin. ACTH secretion depends on the combined releasing activity of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from neuroendocrine cells of the paraventricular nucleus (PVN; Kalsbeek et al. 2006a, 2011b, 2012). The PVN receives indirect projections from the pacemaker and CRH's release is linked to the activity of vasopressin neurons in the SCN (Kalsbeek et al. 2012). This pathway relies on the direct innervation of GABAergic interneurons in the sPVN, a small subnucleus ventral to the PVN (Kalsbeek and Fliers 2013). Thus, the neuroendocrine network regulating GC secretion has been mapped and the physiological control of GC secretion by the SCN is well known (Kalsbeek et al. 2012). In addition to the direct stimulation by circulating ACTH, the timing of GC secretion appears to depend largely on adrenal sensitivity to this peptide hormone, a rhythm dictated by SCN-driven sympathetic cues (Ishida et al. 2005; Oster et al. 2006b). Together these data reveal that SCN-dependent signaling is the primary timing cue for GC secretion, whether through direct neuroendocrine stimulation or indirectly through modulation of sensitivity by autonomic nervous input. The question of clock function outside the SCN within the HPA axis (i.e., PVN neuron and pituitary gland) remains largely unanswered. Currently, there is little to no evidence for circadian clock function in ACTH-secreting corticotrophs or CRH-releasing neurons in the PVN. Though circadian clock gene expression has been detected in the PVN, this expression has not been explicitly localized to CRH neurons (Abe et al. 2002; Shieh 2003). In contrast, evidence does support a role for the adrenal clock in the timing of GC secretion (Oster et al. 2006b; Son et al. 2008; Cailotto et al. 2009; Chung et al. 2011; Torres-Farfan et al. 2011; Kalsbeek et al. 2012; Leliavski et al. 2014).

Most of the known clock genes display robust circadian oscillations in the adrenal cortex (Oster et al. 2006b; Bittman et al. 2003). Rhythms of clock-controlled gene expression in the adrenal gland include the ACTH receptor, g-proteins, and other components of the ACTH signaling cascade (Fig. 2.4; Oster et al. 2006a). Further, rhythms of steroidogenic enzymes including the cholesterol transporter steroidogenic acute regulatory protein (StAR) and the side chain cleavage enzyme Cyp51 have been detected in adrenal cells (Son et al. 2008; Kalsbeek et al. 2012). Thus, the timing of sensitivity to ACTH and the magnitude of the GC secretory response may be dictated in part by the activity of the clock in the adrenal gland. Rhythms of clock gene expression in the adrenal persist in hypophysectomized rats (Fahrenkrug et al. 2008), suggesting that clock function does not depend

on rhythmic ACTH secretion. However, SCN lesions and global clock gene mutations disrupt the rhythm of CORT secretion (Nader et al. 2010; Moore and Eichler 1972), as does the direct inhibition of pacemaker function with clock gene antisense oligonucleotide injections in the SCN (Sellix et al. 2006). These data suggest that the adrenal gland, through a peripheral oscillator, may still depend on central cues to maintain circadian rhythms of GC secretion. To address this issue, Oster and colleagues transplanted adrenals from *per2*/*cry1* double knockout mice into wild-type littermates and vice versa (Oster et al. 2006b). Adrenal glands from WT mice produced a circadian rhythm of serum CORT in animals lacking a functional SCN. These transplant studies, like similar analyses done with the SCN itself (Ralph et al. 1990; Silver et al. 1990), support the physiological autonomy of a self-sustained circadian oscillator in the adrenal. These mice failed to produce a circadian rhythm of CORT secretion when released into constant darkness, indicating that light-driven autonomic cues spared by the clock gene mutations are still necessary to drive rhythms of CORT secretion (Oster et al. 2006b). Subsequent studies using targeted clock disruption support the notion that the adrenal contains a circadian clock that may modulate the timing of ACTH sensitivity but does not have the capacity to drive autonomous rhythms of CORT secretion in the absence of light (autonomic) driven cues from the retina (Son et al. 2008). As we shall discuss with regard to the ovarian clock, the timing of circadian rhythms in the endocrine system can depend as much on the rhythmic secretion of neuroendocrine and pituitary hormones as they do on the timing of sensitivity to these hormones at the target endocrine organ (Ungar and Halberg 1962). Thus, the function of peripheral endocrine clocks may be to modulate or refine sensitivity of the target organ to the central cue (release or inhibiting; neural or humoral), in essence providing the peripheral endocrine organ with the ability to make "predictive" changes in cellular physiology and gene expression in anticipation of the signal (Moore-Ede et al. 1976; Fig. 2.4). To truly understand this system it will be necessary to define the mechanism and functional requirement of each pathway (humoral vs. neural) for maintaining hormonal rhythms (Yoder et al. 2014). Only then can we determine to what extent this concept can be applied more generally to clock function in endocrine physiology.

As indicated above, entrainment to the external environment and synchrony among peripheral oscillators within the timing system is dependent on the synergistic activity of humoral and neural cues (see Fig. 2.2; Menaker et al. 2013; Albrecht 2012). Among the many candidates, adrenal CORT has been repeatedly implicated in the process of entrainment and synchronization in the timing system (Balsalobre et al. 2000; Kiessling et al. 2010; Pezuk et al. 2012; Torra et al. 2000; Yamamoto et al. 2005). Adrenalectomy alters the timing of clock gene expression in several peripheral oscillators, including the liver, kidney, pituitary, and cornea (Pezuk et al. 2012). Further, the rate or kinetics of entrainment following a phase shift of the L:D cycle was altered in several peripheral tissues from adrenalectomized rats. Interestingly, many of these effects could be attenuated following CORT replacement in drinking water (Pezuk et al. 2012). Thus, the circadian rhythm of GC secretion, itself regulated by clocks in the HPA axis, plays a

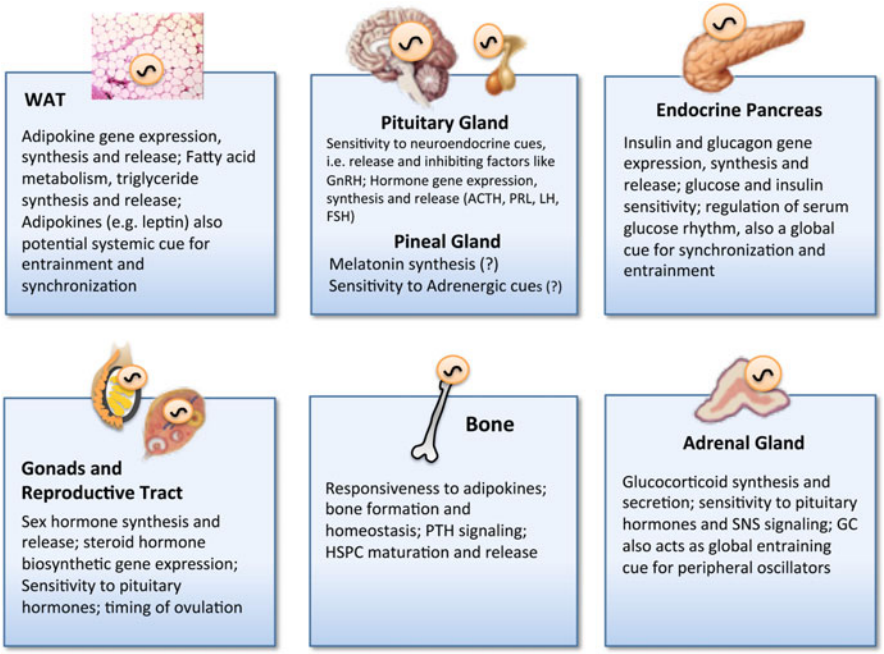

Fig. 2.4 Circadian clock function in endocrine tissues. A summary of the major functions attributed to the circadian clock in each of the major endocrine organs discussed. These functions generally depend in the rhythmic output of the SCN and the timing of clock gene expression in the peripheral tissue. Hormones involved in the process of synchrony and entrainment are highlighted to emphasize their importance to the system. See text for additional details

significant role in the maintenance of synchrony and entrainment within the timing system (Fig. 2.2). CORT acts on target tissues through binding and activation of intracellular GC receptors that have been shown to interact with and regulate the expression of PER and CRY (Yang et al. 2007; Lamia et al. 2011; Balsalobre et al. 2000; Torra et al. 2000). This places CORT in a unique category among hormones, both fundamentally regulated in its secretion by the timing system and simultaneously feeding back on the timing system to modulate and enhance clock function (Fig. 2.2). This feedback function of GCs is not entirely surprising, given the generalized and well-known impacts of hormonal feedback in endocrine physiology. Similar activity may also be applied to other hormones, including pineal melatonin and the adipokine leptin (Carr et al. 2003; Stehle et al. 2003; von Gall et al. 2005; Buijs et al. 2006; Pevet and Challet 2011).

2.5 Circadian Clock Function in the Hypothalamo–Pituitary–Gonadal (HPG) Axis

Circadian rhythms in the female (and to a lesser extent male) reproductive system are critical for regular patterns of hormone secretion, germ cell development, and fertility (Fig. 2.4; Boden and Kennaway 2006; Sellix and Menaker 2011; Tonsfeldt and Chappell 2012). The seminal work of Everett and Sawyer confirmed that a "neural timing" mechanism regulated the preovulatory LH surge in female rodents (Everett et al. 1949; Everett and Sawyer 1950). Subsequent studies revealed that SCN neurons regulate the timing of LH secretion via direct control of gonadotropin-releasing hormone (GnRH) neurons in the medial preoptic area (Palm et al. 1999; Miller et al. 2006; Funabashi et al. 2000). These rhythms are dictated by direct monosynaptic connections between AVPergic and vasoactive intestinal polypeptide (VIP)ergic neurons in the SCN and their target neurons in the medial preoptic area (Van der Beek 1996; Van der Beek et al. 1997a, b). While critical for the precise execution of the preovulatory LH surge within a window of opportunity for fertilization during the reproductive cycle in females, the necessity for clock function in male reproductive physiology is less clear. However, evidence for clock function in the HPG axis of both male and female rodents has been reported. In males, the clock regulates both sperm development and testosterone synthesis (Morse et al. 2003; Alvarez et al. 2003, 2008). In general these are considered non-circadian or nonrhythmic clock functions, dependent on the general *trans* activity of the clock genes with indifference to their rhythmicity. In females, the timing and amplitude of the clock in central and peripheral structures of the HPG axis regulates physiological function ranging from hormone secretion to parturition (Sellix and Menaker 2011; Boden et al. 2013). More compelling evidence for the critical nature of clock function in reproductive physiology comes from studies exploring the impact of circadian disruption (e.g., shift work) on fertility (Kennaway 2005; Kennaway et al. 2012; Gamble et al. 2013; Mahoney 2010; Chau et al. 2013). Chronic shift work is associated with an increased risk of abnormal menstrual cycles, increased time to pregnancy, risk of endometriosis, and miscarriage among women (Chau et al. 2013). Together with data from rodents, these findings confirm the critical function of the timing system in reproductive physiology.

2.5.1 Clock Function in Male Reproductive Physiology

Plasma testosterone levels vary across the day, showing a biphasic pattern with peaks during the rest period and at the onset of activity in humans, rats, and mice (Luboshitzky et al. 2001, 2003; Miyatake et al. 1980; Waite et al. 2009; Lucas and Eleftheriou 1980). Efforts to characterize circadian clock function in the mammalian testis have yielded mixed, and in some cases conflicting, results (Bittman

et al. 2003; Morse et al. 2003; Alvarez et al. 2003, 2008; Alvarez and Sehgal 2005). Morse and colleagues did not detect a circadian rhythm of *per1* or *bmal1* gene expression in mouse testis (Morse et al. 2003). Clock gene expression in sperm cells appears developmentally regulated, with *per1* mRNA elevated in spermatids relative to earlier (spermatogonia) and later (spermatocyte) stages of sperm development (Morse et al. 2003). Further, *clock* mRNA was not colocalized with *per1* during development and *per1* expression in testes was not influenced by the dominant negative *clock* mutation. Two independent studies using male C57BL6/J mice (Alvarez et al. 2003) and BALB/c mice (Bittman et al. 2003) came to similar conclusions regarding the clock in sperm cells. *Per1* mRNA is not rhythmically expressed in seminiferous tubules from either BALB/c or C57BL/6 mice (Bittman et al. 2003). *Per1* mRNA, but not *Per2,* was detected in seminiferous tubules (primarily spermatids and spermatocytes) and interstitial cells (Bittman et al. 2003). Subsequent reports have confirmed that a circadian oscillator (at least one dependent on the rhythmic expression of the canonical clock genes) is not required for normal sperm development (Yamamoto et al. 2004) in mammals.

Though it may not be a critical factor in sperm development, motility, and fertilization capacity given the current evidence, the circadian clock may still play a role in testicular androgen synthesis (Alvarez et al. 2008). Alvarez and colleagues have suggested that the circadian clock regulates fertility in male mice by controlling, in part, the level of steroidogenic enzymes in testicular Leydig cells (the primary testosterone secreting cell in the testes; Alvarez et al. 2008). *Bmal1* knockout mice ($bmal1^{-/-}$) are infertile with significantly reduced sperm counts, though they display normal sperm motility and capacity for fertilization (as assessed by in vitro fertilization). In addition, $bmal1^{-/-}$ mice have lower testosterone and FSH levels and higher LH levels compared with WT mice, suggesting a primary deficit in testosterone synthesis (Alvarez et al. 2008). The expression of several steroidogenic enzymes (e.g., 3-β-hydroxysteroid dehydrogenase) and the primary sterol carrier protein StAR is reduced in the testes of $bmal1^{-/-}$ mice. Surprisingly, a circadian rhythm of BMAL1 protein expression was found by these investigators in both the cytoplasm and nucleus of Leydig cells in wild type mice, in contrast to previous studies (Bittman et al. 2003; Morse et al. 2003). These discrepancies may have arisen from methodological issues related to the lack of cell-type specificity in previous studies. Nonetheless, these data suggest that the circadian clock does not play a role in sperm motility and fertilization capacity, though it may have a function in spermatogenesis. Moreover, the circadian clock in testicular Leydig cells may control the timing and amplitude of androgen synthesis and secretion.

2.5.2 Circadian Clock Function in Female Reproductive Physiology

The female reproductive system exhibits robust circadian rhythmicity, which is normally kept tightly entrained to the light:dark cycle (Fig. 2.4; Kennaway 2005; Boden and Kennaway 2006). In mammals, serum LH and follicle-stimulating hormone (FSH) levels oscillate with a diurnal rhythm on the day of ovulation (Bronson and Vom Saal 1979; Goldman 1999; Legan and Karsch 1975; Moenter et al. 2003). In rodents, the preovulatory LH surge occurs between the latter half of the light portion of the L:D cycle and the early portion of the dark phase or activity period (Bronson and Vom Saal 1979; Everett and Sawyer 1950). This rhythm is dependent upon the circadian clock in the SCN (Moenter et al. 2003; Funabashi et al. 2002; Mitsushima et al. 2003; Wiegand et al. 1980) and free-runs in constant conditions (Mahoney et al. 2004; Stetson and Anderson 1980). In women the LH surge occurs in the late night/early morning on the 10–14th day of the menstrual cycle at the end of the follicular phase and is followed roughly 24–36 h later by ovulation (Kerdelhue et al. 2002; Sellix 2013). Thus, the timing of pituitary gonadotropin release and ovulation are driven by the circadian timing system and are a common feature of mammalian reproductive physiology across species.

It is widely believed that the proestrus LH surge is necessary and sufficient for ovulation, luteinization, and the subsequent formation of a functional corpus luteum (Karsch et al. 1997; Moenter et al. 2003). In rodents, the timing of follicle rupture, limited to a "critical period" on the evening of proestrus, depends on the timing of LH secretion (Kriegsfeld and Silver 2006; Moenter et al. 2003; Karsch et al. 1997). In this view, the ovary is a passive responder to the timing of central endocrine cues. Nevertheless, it has been suggested that the LH surge must come at the appropriate circadian time to be effective at inducing ovulation (Sellix et al. 2010). These data challenge the notion that peripheral oscillators in the female HPG axis are passive with regard to the timing of key events during the reproductive cycle. This concept parallels the discovery in the adrenal gland, again supporting the notion that the circadian clocks in peripheral endocrine organs might play an active role in the timing of sensitivity to their respective activating or inhibiting hypophysiotropic factor. To address this hypothesis, it is first necessary to define the autonomous nature of the clock in tissues of the female reproductive tract. This should be followed by a thorough analysis of potential CCG expression and clock-dependent physiology in each tissue, specifically as it relates to the sensitivity of the oscillator to rhythmic central and hypophysiotropic factors. As detailed below, exciting research has begun to define the physiological role of the clock in tissues of the HPG axis using such an approach. Someday, soon we may have a very clear understanding of the mechanism whereby the clock regulates HPG function and thus the contribution of the timing system to fertility in women.

As in several tissues, the presence of the molecular oscillator, while it belies functional significance, is not always clearly linked to physiology. Early evidence from isolated pituitary explants and pituitary cell cultures indicated that individual pituitary gonadotropes might be semiautonomous circadian oscillators (Lewy

et al. 1996, 1999). In fact, several studies have described cell-autonomous clock gene expression in the pituitary gland (Yamazaki et al. 2000; Abe et al. 2002; Yoo et al. 2004; Leclerc and Boockfor 2005; Resuehr et al. 2007, 2009; Shieh 2003). However, evidence for clock function in specific hormone secreting cells is limited to the gonadotropes and lactotrophs (Leclerc and Boockfor 2005; Bose and Boockfor 2010; Resuehr et al. 2007, 2009). It was determined that the primary hypothalamic GnRH induces *per1* expression in gonadotroph cell lines (Kakar et al. 2003). Others have reported that *per1*, but not *per2*, mRNA expression was activated by GnRH receptor through MAP kinase-dependent signaling (Olcese et al. 2006). In fact, seven E-box sequences in the mouse GnRH receptor (GnRH-R) promoter have been identified and it was reported that BMAL1:CLOCK dimers enhance GnRH-R expression (Resuehr et al. 2007). Further suppression of *bmal1* mRNA expression with siRNA effectively reduced GnRH-R expression. Moreover, GnRH-mediated activation of early growth response protein-1 (EGR-1) also led to enhanced *per* gene expression (Resuehr et al. 2009). Though supportive of a functional role for the clock in these cells, particularly in response to GnRH, subsequent studies have failed to confirm functional necessity of the gonadotroph clock in HPG function. Gonadotroph-specific deletion of *bmal1* had little effect on the amplitude and timing of LH and FSH secretion and these transgenic mice are fertile, though the duration of their reproductive cycles was increased (Chu et al. 2013). These data suggest that molecular clock function in regions upstream [basal hypothalamus, GnRH neuron (Chappell 2005)] or downstream [ovary (Sellix 2013)] may be more critical for normal reproductive function in mice.

Rhythms of clock gene expression have been found in the whole ovary (Fahrenkrug et al. 2006; Karman and Tischkau 2006; He et al. 2007a, b; Tischkau et al. 2011) and isolated ovarian granulosa cells (He et al. 2007b; Chu et al. 2011, 2012; Chen et al. 2013a, b; Yoshikawa et al. 2009) from both rats and mice. Two independent studies reported rhythms of clock gene expression in the rat ovary (Karman and Tischkau 2006; Fahrenkrug et al. 2006). Gonadotropin exposure in vivo induced cyclic expression of *bmal1* and *per2* mRNA in the ovaries of hypophysectomized prepubertal rats (Karman and Tischkau 2006). Further, diurnal rhythms of *per1* and *per2* expression were observed that persisted across the reproductive cycle (Fahrenkrug et al. 2006). Rhythms of clock gene expression were also detected in large pre-antral follicles, small antral follicles, Graafian follicles, and corpora lutea. Reports indicate that rhythms of clock gene expression are limited to mature isolated granulosa and luteal cells (He et al. 2007a, b; Chu et al. 2011, 2012). The physiological significance of rhythmic clock gene expression in the ovary remains unknown. It is possible that the ovarian clock modulates the timing of ovulation or regulates the timing and amplitude of ovarian steroid and peptide hormone secretion. It has been suggested that the timing of ovulation may depend on rhythmic sensitivity of the ovary to gonadotropins (Sellix et al. 2010; Sellix 2013). While these data indicate that the clock in the ovary could drive rhythms of sensitivity to LH, there is little evidence supporting the cell-autonomous nature of this sensitivity in follicular cells. It is well known that the LH surge induces a significant change in gene expression within the granulosa and thecal cells lining the preovulatory follicle (Espey and Richards 2002; Espey et al. 2003;

Richards 2005). However, it remains to be seen if these LH-responsive genes oscillate with a circadian rhythm in the absence of the surge. Several candidate genes associated with the response of the granulosa and thecal cells to LH can be considered CCGs, including prostaglandin synthase, LH receptor, steroidogenic enzymes, gap junction proteins, and transcription factors like liver receptor homolog-1 (*lrh-1*; Chen et al. 2013a, b; Sellix and Menaker 2010).

A significant step in the response of the ovarian granulosa cell to LH is the increase in the level of prostanoids, including prostaglandin E2 (PGE2) and PGF2α (Sirois et al. 2004). The expression of cyclooxygenase-2 (COX2), the rate-limiting enzyme for prostaglandin synthesis, is regulated by E-box promoter elements (Sirois et al. 2004). These data suggest that successful follicular rupture, a process dependent on prostanoid signaling, may require proper transcriptional control by the circadian clock. Another likely candidate is liver receptor homolog-1 (LRH-1), which was first identified as an orphan nuclear receptor in the liver (Nitta et al. 1999). In the ovary, *lrh-1* expression is limited to the granulosa cell layer (Liu et al. 2003). In the liver, LRH-1 binds to the core circadian clock protein CLOCK (Oiwa et al. 2007) and acts synergistically to drive CLOCK-BMAL1-mediated transcription (Oiwa et al. 2007). Whether LRH-1 has the same activity in ovarian cells is currently unknown. In the ovary, LRH-1 controls steroid hormone biosynthesis in granulosa cells through direct activation of cytochrome P450 side chain cleavage (*cyp11a1*) transcription following luteinization (Kim et al. 2005). It has been suggested that regular *lrh-1* expression in mouse granulosa cells is critical for ovulation (Duggavathi et al. 2008). It remains to be seen if disruption of core molecular clock function in mouse granulosa cells results from, or is linked to, abnormal *lrh-1* or *cox2* gene expression. Nevertheless, the reduced fecundity associated with *lrh-1* deficiency and the link between *lrh-1* or *cox2* transcription and core clock gene expression make it likely that disruption of the circadian clock in the ovary will have detrimental effects on fertility. Treatment with PGE2 in vivo has been shown to phase shift the rhythm of *per1*, d-element binding protein *(dbp)*, and Rev-erbα (*nr1d1*) mRNA expression in the heart, liver, and kidney (Tsuchiya et al. 2005). Most recently, it was revealed that luteinized or "mature" granulosa cells do in fact have robust circadian rhythms of *ptgs2* and LH receptor *(lhcgr)* gene expression that are disrupted and in some cases abolished following attenuation of *bmal1* expression (Chen et al. 2013b). Together, these data suggest that an increase in COX2 and LHCGR expression and/or PG activity preceding the arrival of the LH surge may allow for predictive changes in ovarian cells in anticipation of ovulation. New evidence suggests that the appearance of robust rhythms of clock gene expression in mature follicles may be due to FSH-dependent expression of gap junction proteins (Chen et al. 2013a). Disruption of intercellular communication via gap junction blockers reduces the amplitude and lengthens the period of PER2 expression in rat granulosa cells (Chen et al. 2013a). The suggestion being that gonadotropin-dependent communication among follicular cells may play a critical role in the appearance and/or maintenance of clock-controlled gene expression in ovarian follicular cells. Identifying a link between the timing of the circadian clock, the timing of ovarian sensitivity to LH, and the expression of CCGs like lrh-1 and

gap junction proteins, may lead to improved understanding of the molecular basis for common and devastating causes of infertility. Evidence from rodents (Chen et al. 2013a, b; Alvarez et al. 2008; Ratajczak et al. 2009) and birds (Nakao et al. 2007) suggests that the circadian clock plays a significant role in the amplitude and timing of steroid hormone biosynthesis. Circadian rhythms of StAR, 3-beta-hydroxysteroid dehydrogenase (3β-HSD), 11α-hydroxylase, and aromatase (cyp19) have been observed in mature granulosa cells (Chen et al. 2013a, b). These rhythms are altered or abolished following clock disruption (Chen et al. 2013b). Further, *bmal1*$^{-/-}$ mice have abnormally low levels of progesterone secretion due to reduced StAR protein expression (Ratajczak et al. 2009). Overall, the evidence for autonomous clock function in follicular cells supports a functional role for the clock in ovarian steroid synthesis, follicular growth, and ovulation. Finally, it has been reported that mice with targeted deletion of *bmal1* in the steroidogenic cells of the ovary are infertile (Liu et al. 2014). In this study it was determined that deletion of *bmal1* in all steroid-producing cells of the ovary using a Steroidogenic-Factor 1-Cre transgenic mouse (*SF1-CRE;* *bmal1*$^{FLX/FLX}$) resulted in severely compromised luteal progesterone (P4) secretion. This drop in P4 led to implantation failure and infertility, most likely due to a decline in BMAL1:CLOCK-driven steroidogenic factor expression, including StAR. Using an elegant ovarian transplant approach, the investigators confirmed that this effect was limited to ovarian progesterone production and not indirect effects of SF1-CRE-mediated *bmal1* deletion in other tissues (Liu et al. 2014).

Clock genes are expressed in the rodent oviduct (Johnson et al. 2002; Kennaway et al. 2003). Rhythms of *per2, bmal1, dbp, pai1* (plasminogen activator inhibitor-1; PAI-1), and *n1rd1* (REV-ERBα) have been described in oviduct epithelium, suggesting that the embryo is exposed to rhythmic "environmental conditions" during transition from the ovarian bursa to the uterus (Kennaway et al. 2003). It has been suggested that rhythmic secretory activity of oviduct epithelial cells may be critical for embryonic development, though evidence is limited. As with the ovary, additional functional studies of clock-dependent physiology are needed to confirm the role of the clock in the oviduct. In the uterus, the circadian clock has been implicated in the process of implantation, development of the conceptus, and parturition (Ratajczak et al. 2009, 2010, 2012; Johnson et al. 2002). As in the ovary and oviduct, evidence reveals that uterine cells are semiautonomous circadian clocks (Johnson et al. 2002; He et al. 2007a; Nakamura et al. 2005; Akiyama et al. 2010). The timing of clock gene expression in the uterus appears to be affected by variations in the ovarian steroid hormone secretion across the reproductive cycle (Nakamura et al. 2010) and direct stimulation with ovarian steroids (Hirata et al. 2008; Nakamura et al. 2008). *Bmal1* knockout mice display reduced fertility marked by altered levels of steroid hormone synthesis and implantation failure (Ratajczak et al. 2009). Further, targeted deletion of *bmal1* gene expression in the uterine myometrium disrupts normal implantation (Ratajczak et al. 2012). In summary, the circadian clock plays a significant role in ovarian and uterine physiology. Though data suggest that the clock is linked to physiological function in the oviduct and pituitary gland, evidence is limited.

2.6 Circadian Clock Function and the Endocrine Control of Bone Homeostasis

Homeostatic control of bone structure relies on counteracting activity of bone forming osteoblasts and bone metabolizing or resorbing osteoclasts (Rodan and Martin 2000). Circadian rhythms of bone metabolism have been well described, though a mechanism for this oscillation has yet to be fully characterized (Fig. 2.4; Hart and Eastell 1999; Bjarnason and Jordan 2000; Giudice et al. 2010; Kawai et al. 2010; Kawai and Rosen 2010; Hinoi et al. 2006; McElderry et al. 2013). Daily rhythms of extracellular matrix proteins including type I collagen and osteocalcin have been reported in bone (Simmons and Nichols 1966; Gundberg et al. 1985). A circadian rhythm of bone mineralization has been described in neonatal calvarial bone explants, suggesting that bone formation is controlled by the molecular clock (McElderry et al. 2013). Clock gene expression has been observed in bone marrow and hematopoetic stem cells (Mendez-Ferrer et al. 2008; Chen et al. 2000). Further, bone mass is enhanced in clock gene mutant animals due to deficits in leptin-dependent sympathetic inhibition of bone formation by osteoblasts (Fu et al. 2005). This effect of clock gene mutation is linked to enhanced cyclin expression in osteoblasts. These data suggest that the adipokine leptin, acting through β-adrenergic receptors, can both enhance and suppress osteoblast proliferation. Thus, the back and forth between bone ossification and resorption depends on the timing of adipokine secretion, rhythmic sympathetic signals, and local control of bone cell metabolism by the molecular clock in osteoblasts and osteoclasts. In addition to SCN-dependent autonomic nervous cues, the timing of bone metabolism may also be regulated by circulating parathyroid hormone (PTH).

PTH is an anabolic hormone secreted by Chief cells of the parathyroid gland (Hanyu et al. 2011). A daily rhythm of serum PTH in humans was first described in the early 1970s (Fraser et al. 2004). PTH levels generally increase during the late night and peak in the early morning 2–3 h before waking (Jubiz et al. 1972). The timing and amplitude of PTH synthesis and secretion from Chief cells depends on the level of ionized calcium in extracellular fluid, a response mediated by membrane bound calcium-sensitive receptors. It has also been suggested that circadian rhythms of PRL and GC secretion may regulate the timing of PTH secretion. The rhythm of PTH can be altered by sleep–wake cycles and food intake, supporting the notion that it may also be driven, in part, by the circadian clock in Chief cells (Jubiz et al. 1972; Czeisler and Klerman 1999). Unfortunately, circadian clock gene expression has yet to be described in isolated Chief cells. Though clock function in these cells remains largely undefined, there is considerable evidence supporting a role for the clock in PTH signaling at target cells in bone. PTH increases PER1 expression via a MAPK-CREB-dependent mechanism in osteoblasts and that PER1 is necessary for the action of PTH on these cells (Hanyu et al. 2011; Hinoi et al. 2006). Thus, PTH can affect the timing of the clock in osteoblasts and the clock appears necessary for normal PTH signaling in bone. The importance of rhythmic PTH secretion as it relates to bone metabolism and the clinical

ramifications of altered PTH temporal secretion remain unknown. As does the potential advantages of phasic PTH exposure or adjusting the sensitivity of the bone clock to PTH signaling. Further, the putative role of PTH as a synchronizing and entraining cue, not only for bone cells but across the entire body, remains a mystery. It is worth noting, however, that PTH is provided clinically with little regard for clock function in target tissues and that chronotherapy in the treatment of bone disease is an area in need of additional investigation (Dhillon et al. 2013; Kaur et al. 2013). In summary, the influence of the timing system on bone homeostasis conforms nicely to a recurrent theme in chronophysiology: the coordination of divergent but critical physiological functions by synergistic input from central and peripheral oscillators.

2.7 Circadian Clock Function in the Mammalian Pineal Gland

One of the most well-studied and thoroughly described endocrine rhythms is the daily pattern of pineal melatonin secretion (Kennaway and Wright 2002; Stehle et al. 2003; Scheer and Czeisler 2005). The majority of what we know about melatonin relates to its role in seasonal rhythms of reproductive function or so-called photoperiodism (Reiter 1980; Seamark et al. 1981; Goldman and Darrow 1983; Karsch et al. 1984; Kennaway 1988). In addition to this function, melatonin has also been described as a potent antioxidant and a significant factor in the etiology of complex metabolic diseases like diabetes (Peschke 2008; Suzen 2013). Pineal melatonin is secreted from pinealocytes during the night (in both nocturnal and diurnal mammals) and is regulated by a complex neuroendocrine circuit originating with SCN pacemaker neurons (Fig. 2.4; Kalsbeek et al. 2006b; Buijs et al. 2006; Borjigin et al. 2012). SCN neurons balance the activity of autonomic neurons in the PVN, leading to increased adrenergic signaling in the pineal during the night and suppressed signaling during the day (Borjigin et al. 2012). In addition to central control by the SCN, evidence suggests that the molecular clock in pinealocytes could play a role in the timing and amplitude of melatonin secretion in mammals (Nishide et al. 2014; Christ et al. 2010; Ansari et al. 2009; Ackermann et al. 2007; Chansard et al. 2005, 2006; Karolczak et al. 2005). Unlike mammals, the role of the molecular clock in pineal melatonin secretion has been more extensively described in lower vertebrates, including both fish and avian species (Nagy et al. 2009a, b; Karaganis et al. 2008; Zilberman-Peled et al. 2007; Ziv and Gothilf 2006; Yasuo et al. 2003; Whitmore et al. 1998, 2000). In rodents, clock gene expression rhythms have been characterized in the mouse (Karolczak et al. 2004; von Gall et al. 2001; Stehle et al. 2001), rat (Yoshikawa et al. 2005; Simonneaux et al. 2004; Shieh 2003; Fukuhara et al. 2002; Takekida et al. 2000), and hamster (Johnston et al. 2003) pineal gland.

Though it was long believed that the pinealocyte was not a self-sustained cell-autonomous circadian oscillator (Karolczak et al. 2004; Maronde and Stehle 2007;

Simonneaux et al. 1989), having lost this function along with its direct photic sensitivity, more recent evidence refutes this claim (Pezuk et al. 2010; Wongchitrat et al. 2009; Christ et al. 2010). Nonetheless, a role for the molecular clock in the mammalian pineal gland, specifically as it relates to melatonin biosynthesis and release, remains controversial (Maronde and Stehle 2007). When isolated from SCN input, rhythms of pineal melatonin secretion are lost (Simonneaux and Ribelayga 2003). Rhythms of Per1-luciferase and Per2-luciferase transgene expression were present in isolated pineal tissue cultures but appeared to disappear within 2–3 days (Abe et al. 2002; Nishide et al. 2014). Nonetheless, the rhythmic expression of PER1 appears to be linked to the timing of norepinephrine (NE)-dependent upregulation of arylalkylamine *N*-acetyltransferase (AANAT) gene expression, a rate-limiting step in melatonin biosynthesis (Drijfhout et al. 1996a, b; Fukuhara et al. 2005). It is possible that this oscillation may simply be linked to cAMP-dependent signaling in the pinealocyte (Karolczak et al. 2004). Further, the rat AANAT gene promoter contains an E-box, the canonical DNA binding motif for clock gene enhancers, and the CLOCK:BMAL1 complex can activate Per1 mRNA expression in isolated pinealocytes (Chen and Baler 2000; Fukuhara et al. 2002). Moreover, *bmal1* and *nr1d1* (Rev-erbα) mRNA expression oscillates in a NE-independent fashion in the hamster pineal (Wongchitrat et al. 2009). These data suggest that the synthesis and secretion of pineal melatonin may depend as much on indirect noradrenalin-dependent signaling from SCN-derived autonomic input as it does on local control of gene expression in the pinealocyte.

As an endocrine hormone, melatonin has a multitude of peripheral and central targets including the SCN, liver, ovary, testes, pituitary, and endocrine pancreas (Peschke et al. 2007; Peschke 2008; Peschke and Muhlbauer 2010). In the endocrine pancreas, melatonin influences insulin secretion via both the type 1 and type 2 melatonin receptors on β-cells (Peschke 2008). Serum melatonin levels are lower among patients with type-2 diabetes (T2D) and melatonin treatment appears to improve glucose homeostasis and insulin sensitivity in a mouse model of T2D (Sartori et al. 2009; Srinivasan et al. 2013). Thus, in addition to its putative role in the timing and amplitude of sleep and seasonal reproduction, melatonin also appears to play a considerable role in glucose metabolism and insulin tolerance, further strengthening the connection between chronodisruption, metabolism, and endocrine disease (McMullan et al. 2013a, b; Rubio-Sastre et al. 2014). The finding that sleep disturbances, including sleep apnea, are more common among patients with T2D further supports the relationship between sleep quality, glucose homeostasis, and pineal melatonin secretion (Laposky et al. 2008b; Mantele et al. 2012; Aurora and Punjabi 2013). Conflicting evidence from Garaulet and colleagues indicates that melatonin treatment could worsen glucose tolerance, particularly in the early morning (Rubio-Sastre et al. 2014; Garaulet et al. 2015). Further, these studies suggest that a fairly common genetic variant of the MNTR1B gene that codes for the MT2 melatonin receptor increases the risk of developing glucose intolerance following oral melatonin treatment. It remains to be seen whether regular treatment with melatonin or synthetic melatonin receptor agonists, such as ramelteon (rozerim), are effective adjunct therapies for improving insulin resistance, glucose homeostasis, and sleep quality in patients living with T2D.

2.8 Circadian Clock Function and the Timing of Prolactin Secretion

The pituitary peptide hormone prolactin (PRL) has over 300 known physiological functions, playing a role in everything from reproductive physiology to the immune response (for a thorough review, see Freeman et al. 2000). PRL is secreted by lactotrophs or somatomammotrophs located within the adenohypophysis or anterior lobe of the pituitary gland. PRL secretion is regulated by multiple hypophysiotropic releasing and inhibiting hormones including oxytocin, thyroid stimulating hormone, and dopamine (DA; Ben-Jonathan 1985; Ben-Jonathan et al. 1989; Samuels et al. 1991; Murai et al. 1989). Hypothalamic PRL regulating factors reach the anterior lobe via two main routes: through the primary long portal vessels draining the capillary beds of the median eminence or through short portal vessels connecting the posterior or "neural lobe" to the anterior lobe (Ambach et al. 1976; Mezey et al. 1982; Palkovits 1992; Palkovits et al. 1998). Dopamine (DA) of hypothalamic origin exerts tonic inhibitory control over PRL secretion and is considered the primary PRL regulatory factor (Ben-Jonathan 1980, 1985). Three populations of neuroendocrine DAergic neurons (NDNs) in the hypothalamus release DA into portal vasculature, including the tuberoinfundibular dopaminergic (TIDA), tuberohypophyseal dopaminergic (THDA), and periventricular hypophyseal dopaminergic (PHDA) neurons (Freeman et al. 2000). Though the TIDA neurons are thought of as the primary PRL inhibitory neurons, a growing importance has been assigned to both the THDA and PHDA neurons (Mai et al. 1994; DeMaria et al. 1998b). In fact, evidence from posterior lobectomy experiments strongly supports a role for the THDA and PHDA in the regulation of PRL secretion (Murai and Ben-Jonathan 1986, 1987). Though each has been linked to the circadian timing system, PRL secretory patterns are heavily dependent on the physiological status of the animal. During the estrous cycle, a surge of PRL secretion occurs on the afternoon of proestrus, in response to positive feedback from rising titers of ovarian steroids (Freeman et al. 1988). Experiments with ovariectomized (OVX) rats given exogenous steroid treatment suggest a critical period for the timing of the PE rise in serum PRL, very similar to that timing of LH secretion (Neill 1972). A single bolus injection of estrogen, given to OVX rats, will induce daily surges of PRL secretion around 1600 h, suggesting a circadian regulatory system modulated by steroids. DA levels in the median eminence and pituitary gland, as well as the activity of DA synthetic enzymes in the arcuate nucleus, decline during the proestrous surge (Demarest et al. 1981; DeMaria et al. 1998a). The neuroendocrine reflex driving PRL secretion during lactation is one of the most well-characterized hormone rhythms in mammalian physiology (Mena and Grosvenor 1972; Blake 1974). Serum PRL levels increase within minutes of the suckling stimulus, remain elevated during nursing, and are proportional to the intensity of the nursing stimulus, i.e., the number of pups. The magnitude of PRL secretion in response to suckling increases in the afternoon, suggesting a synergism between the neuroendocrine reflex and the circadian timing system (Kacsoh and Nagy 1983;

Arey et al. 1991). Finally, stimulation of the uterine cervix which leads to pregnancy or pseudopregnancy (PSP) induces a PRL secretory pattern characterized by a nocturnal surge (N) between 0100 and 0500 h and a diurnal surge (D) occurring between 1600 and 1800 h (Gunnet and Freeman 1983). The two daily surges of PRL initiated after application of a mating stimulus to the uterine cervix provide the primary luteotrophic support necessary to maintain the progesterone secretory activity of the corpus luteum during early pregnancy. These daily surges of PRL during PSP persist for 10–12 days and are driven by synergistic input from both hypothalamic releasing and inhibiting hormone (Egli et al. 2004). Stimulation of the uterine cervix results in a significant decrease in the activity of the NDNs at the approximate time of each surge (Lerant et al. 1996). Evidence suggests that the timing of the two daily PRL surges during PSP is mediated by direct input from the SCN (Bethea and Neill 1980). The current model suggests that VIP containing SCN neurons (primarily in the ventrolateral portion of the nucleus) signal timing cues to oxytocinergic neurons in the PVN. Oxytocin (OT) acts indirectly on interneurons within the hypothalamus in order to decrease DAergic tone (Egli et al. 2004). In addition to regulating DA release indirectly through OTergic neurons, VIP-containing nerve terminals from the SCN also terminate directly on NDNs (Van der Beek et al. 1997a; Horvath 1997; Gerhold et al. 2001). This role for VIPergic SCN neurons is not novel, as their participation in the timing of pituitary gonadotropin secretion is well established (Levine 1997). In fact, injection of VIP antisense into the SCN has been shown to disrupt LH secretion and a decline of rhythmic VIP release in aging females has been linked to reproductive senescence (Krajnak et al. 1998, 2001, 2003; Harney et al. 1996; Wise et al. 1997). Likewise, disruption of VIP expression in the SCN modulates the timing and magnitude of immediate early gene expression in NDNs (Gerhold et al. 2002). Together, these data support the notion that VIPergic SCN neurons project to and regulate the timing of DA release from the basal hypothalamus.

As noted above, the circadian timing system is considered a network of synchronized central and peripheral oscillators, with the SCN reigning as pacemaker for peripheral clocks (Menaker et al. 2013). While certainly prevalent in the periphery, clock gene expression has been observed in many of the brain regions outside of the SCN, including multiple regions of the forebrain, thalamus, and hypothalamus (Kriegsfeld et al. 2003; Abe et al. 2002; Guilding et al. 2009). Many of the brain regions that receive direct neural input from the SCN also display cell-autonomous rhythms of clock gene expression when isolated (Guilding and Piggins 2007; Guilding et al. 2009, 2010; Granados-Fuentes et al. 2004; Abraham et al. 2005). Given the multitude of data suggesting that neural targets of the SCN, like the mediobasal hypothalamus, may express functional clock genes, it was suggested that clock gene expression in NDNs might play a role in the timing of PRL secretion. Light-entrained and free-running circadian rhythms of clock gene expression are present in the basal hypothalamus, specifically in the NDNs located in the arcuate nucleus (Guilding et al. 2009; Sellix et al. 2006). Disruption of clock gene expression within the SCN following the injection of antisense deoxyoligonucleotides (AS-ODN) in vivo altered, but did not abolish light-entrained circadian

rhythms of DA turnover and PRL secretion (Sellix et al. 2006). The level of total DA present in the anterior pituitary was shifted and increased, leading to a small delay in the onset of the afternoon PRL surge (Sellix et al. 2006). Overall, these data suggest that NDNs are semiautonomous circadian oscillators entrained to the 24L: D cycle by the SCN but with the capacity to maintain daily rhythms of DA release and PRL secretion in the absence of pacemaker input. It is worth noting that the suppression of clock function in these experiments was transient (less than 48 h), was incomplete (only a 50–70 % reduction in protein levels), and failed to target the *bmal1* gene. Additional experiments targeting clock gene mutation to specific hypothalamic neuroendocrine cells (e.g., using a CRE-LOX system) in vivo are needed to more completely define clock function in these cells.

2.9 The Circadian Timing System and Endocrine Control of Metabolism

The timing system regulates daily rhythms of lipid and glucose metabolism (Green et al. 2008; Bass and Takahashi 2010; Huang et al. 2011). A majority of the major hormones involved in energy balance oscillate with a circadian rhythm including insulin (La Fleur et al. 1999), adiponectin (Ando et al. 2005), glucagon (Ruiter et al. 2003), and leptin (Bodosi et al. 2004; Kalsbeek and Fliers 2013). The rhythmic activity of each of these hormones is linked both to the timing of feeding behavior, nutrient intake and tissue specific regulation of hormone biosynthesis, and secretion (Prasai et al. 2011; Kalsbeek et al. 2011a; Morris et al. 2011; Kalsbeek and Fliers 2013). Circadian disruption, environmental (e.g., shift work) or genetic (clock mutation or gene polymorphism), is associated with obesity, hyperinsulinemia, dyslipidemia, and increased risk for T2D and cardiovascular disease (Fig. 2.3; Ruger and Scheer 2009; Scheer et al. 2009; Morris et al. 2011; Green et al. 2008; Arble et al. 2010). The role of the SCN in driving daily rhythms of food intake and glucose metabolism is well established. In mammals, rhythms of circulating glucose and insulin secretion are regulated by the pacemaker (La Fleur et al. 1999; Kalsbeek et al. 2010) and are abolished following SCN lesions (Yamamoto et al. 1987). Further, these rhythms are tightly associated with changes in food intake and behavior (Diaz-Munoz et al. 2000). The daily rise in serum glucose depends on sympathetic and parasympathetic innervation of the liver, but is not influenced by attenuation of the circadian rhythm of cortisol secretion (Bright et al. 1980; Kalsbeek et al. 2008). In addition to rhythms of glucose release, daily changes in glucose tolerance have been reported, marked by a peak in the early portion of the activity period (La Fleur et al. 2001; Shi et al. 2013). With that in mind, it is important to remember that metabolic homeostasis depends on the integration of glucose and lipid metabolism on an organismal scale, a process that most certainly depends on temporal synchrony among a majority of the mammalian peripheral oscillators (e.g., liver, lung, muscle, etc.). Most notable is the potential

role of the clock in the timing of glucose uptake, which is rhythmic in several tissues including skeletal muscle, liver, lung, and adipose tissue (Oike et al. 2010; Feneberg and Lemmer 2004). Thus, coordination of insulin, glucose, and adipokine levels with the phasic response to each represents separate but equal components of metabolic homeostasis. It is also necessary to highlight the role of meal timing in circadian control of metabolism. Food availability is a potent timing cue for peripheral oscillators including the liver, pancreas, and adipose tissue (Diaz-Munoz et al. 2000; Hara et al. 2001; Stokkan et al. 2001; Stephan 2002). Abnormal patterns of food intake can compete with or alter patterns of hormone secretion, leading to misalignment between central control of metabolism and peripheral clock function (Laposky et al. 2008a). Our focus will be on clock function in peripheral oscillators including both the endocrine pancreas (Marcheva et al. 2011) and white adipose tissue (WAT; Johnston et al. 2009) that have been shown to make sizeable contributions to metabolic homeostasis in mammals. These tissues are unquestionably the most well-characterized peripheral oscillators involved in the endocrine control of metabolism (Johnston 2012; Huang et al. 2011; Marcheva et al. 2013).

2.9.1 Circadian Clock Function in the Endocrine Pancreas

Daily rhythms of pancreatic insulin secretion (Kalsbeek et al. 1998; La Fleur 2003; Morris et al. 2011; Green et al. 2008; Bass and Takahashi 2010) and glucagon (Ruiter et al. 2003) secretion are associated with food intake and glucose metabolism. The daily rhythm of serum insulin rises prior to activity and feeding onset in order to prepare for the first meal following an extensive fast (Kalsbeek et al. 1998; Kalsbeek and Strubbe 1998). In contrast, the daily rhythm of glucagon increases near the end of the activity/feeding period (approximately 12 h later) and is abolished following electrolytic lesion of the SCN (Ruiter et al. 2003; La Fleur 2003; Yamamoto et al. 1987). Glucagon secretion is also tightly linked to patterns of food intake in rats (Ruiter et al. 2003). Though also linked to feeding, the daily rhythm of insulin secretion persists during fasting and is not abolished by regular distribution of small meals across the 24-h day (Kalsbeek et al. 1998; Kalsbeek and Strubbe 1998). Like the daily rhythm of glucagon, rhythms of pancreatic insulin secretion are also abolished following destruction of the central pacemaker (Yamamoto et al. 1987). Together, these data indicate differential and complex control of rhythmic insulin and glucagon secretion by the central pacemaker and food intake. In addition to the timing of insulin secretion, the circadian timing system also appears to modulate the timing and amplitude of insulin sensitivity in target organs (Shi et al. 2013; Sauermann et al. 2011; Wang et al. 2011; Oishi et al. 2005). These data are further supported by the evidence that disruption of the circadian timing system alters glucose homeostasis and insulin sensitivity, leading to increased risk of metabolic disease including T2D and cardiovascular disease (Scheer et al. 2009; Kalsbeek et al. 2011a; Buxton et al. 2012). In humans, genetic polymorphisms at

specific clock gene loci are also associated with an increased risk of obesity and T2D (Scott et al. 2008a, b; Woon et al. 2007). Likewise, altered clock function, due to SCN lesion, abnormal lighting conditions, or clock gene mutations in rodents is associated with obesity and metabolic disease (Turek et al. 2005; Preuss et al. 2008; Coomans et al. 2013a, b; Rudic et al. 2004). Conversely, obesity and metabolic disease can alter the timing and amplitude of clock gene expression in various central and peripheral oscillators (Kohsaka et al. 2007; Arble et al. 2009, 2010). This establishes a "chicken and egg" relationship between the circadian timing system and metabolic homeostasis; disruption of the timing system can, either acutely or over time, enhance the risk of metabolic disease; whereas metabolic disease perturbs the clock and enhances disease progression. Definitive evidence that "fixing" a broken clock can restore metabolic function is limited and will require long-term study of animals or human subjects after recovery from chronic or acute periods of circadian disruption. Hope may come from the burgeoning fields of chronotherapeutics and chronopharmacology. It has been suggested that phasic drug therapy or treatment with drugs that target clock gene function may be able to remedy metabolic disease (Burris et al. 2012). As with any new approach to treatment, more focused and well-designed clinical trials are needed before serious consideration is given to these drugs as first-line clinical treatment options.

Daily rhythms of insulin secretion from cultured rat's pancreatic Islets of Langerhans indicated that pancreatic β-cells could be autonomous oscillators (Peschke and Peschke 1998; Csernus et al. 1998; Delattre et al. 1999). As in many tissues, the molecular clock is present in hormone secreting pancreatic cells (Marcheva et al. 2010; Sadacca et al. 2011; Rudic et al. 2004; Vieira et al. 2012, 2013; Allaman-Pillet et al. 2004). Disruption of clock gene expression in pancreatic β-cells reduces insulin secretion, islet size, and survival (Marcheva et al. 2010; Sadacca et al. 2011; Vieira et al. 2012). Targeted deletion of *bmal1* or *clock* expression resulted in severe reduction in islet size and insulin secretion. These effects appeared to be due to disrupted insulin secretory function and not sensitivity of the β-cell to glucose. Studies confirmed altered expression of genes associated with vesicular packaging, docking, and exocytosis following clock disruption in β-cells. The molecular clock also regulates the timing and amplitude of glucagon secretion from pancreatic α-cells. Suppression of *rev-erbα* in alphaTC1-9 cells (a pancreatic α-cell line) attenuates glucagon secretion in a low glucose environment and disrupts expression of proteins involved in exocytosis (Vieira et al. 2013). Though functional evidence is limited, clock genes are also expressed in pancreatic δ-cells. Evidence for circadian clock function in the endocrine pancreas is considerable and indicates that the pancreatic clock plays a significant role in the process of glucose homeostasis. As with the peripheral clocks in the HPG and HPA axis, it remains to be seen if complex neural and humoral pathways act synergistically to regulate the timing of the pancreatic clock. The timing of insulin sensitivity depends on both neural and humoral input driven by the SCN and nutrient cues provided by timed food intake (Yi et al. 2012; Coomans et al. 2013b). The mechanism by which the pancreatic clock regulates sensitivity to these cues remains largely unknown. Nonetheless, clock function in the pancreas is a critical

aspect of metabolic homeostasis. Understanding the details of clock control in the endocrine pancreas may yet provide new insight into the pathogenesis of diabetes and novel approaches to the treatment of obesity and metabolic disease. As an example, it has been shown that treatment with selective RORα targeting drugs can prevent insulitis and the incidence of type-1 diabetes, an effect linked to the influence of these compounds on TH17 cell cytokine release and autoantibody production (Solt et al. 2015).

2.9.2 Circadian Clock Function in Adipose Tissue

The circadian timing system regulates daily rhythms of physiological function in adipose tissue including daily patterns of lipid metabolism, adipocyte proliferation, and adipokine secretion (Fig. 2.4; Gomez-Santos et al. 2009; Otway et al. 2009; Johnston et al. 2009; Johnston 2012; Shostak et al. 2013a, b; Ando et al.). High-throughput genetic screens confirm that a considerable portion of the transcriptome in white and brown adipose is rhythmically expressed in mammals (Zvonic et al. 2006). Energy is stored in WAT in the form of TGs, which can be further broken down to FFA and glycerol by lipolysis. It is necessary to strike a careful balance between periods of fatty acid triglyceride (TG) synthesis and catabolism, in order to prevent excess levels in the circulation, a situation commonly associated with metabolic disease (Shostak et al. 2013a, b; Johnston 2012). Circulating levels of FFA, TG, and glycerol oscillate in a circadian fashion (Shostak et al. 2013b). In addition to rhythms of lipid metabolism, WAT also acts as an endocrine organ, rhythmically secreting adipokines including leptin, adiponectin, and visfatin (Shostak et al. 2013b; Kalsbeek et al. 2001; Rudic et al. 2004; Kalsbeek and Fliers 2013). The timing of rhythmic lipid metabolism and adipokine secretion in WAT is regulated by SCN-driven parasympathetic and sympathetic input (Kreier et al. 2002; Bartness and Bamshad 1998; Bamshad et al. 1998). Of these, the rhythm of leptin secretion has been most extensively characterized in both humans and rodents (Simon et al. 1998; Kalsbeek et al. 2001). Serum leptin levels peak during the inactive or fasting period and are associated with reduced appetite (Schoeller et al. 1997; Harrold et al. 1998; Kalsbeek et al. 2001; Pan and Kastin 2001; Trayhurn 2001; Ruger and Scheer 2009).

Disruption of the central pacemaker attenuates this daily rhythm of leptin secretion in rats (Kalsbeek et al. 2001). Mice with circadian clock mutations (*clock* and *bmal1*) display increased fat mass, larger adipocytes, higher TG, and adipokine levels in serum and blunted rhythms of FFA and glycerol (Turek et al. 2005; Shostak et al. 2013a). Circadian disruption (e.g., shift work, etc.) alters the timing of clock function in adipose tissue, leading to abnormal patterns of adipokine secretion (Husse et al. 2012; Broussard and Brady 2010). It is also notable that mice with leptin deficiency (*ob*/*ob*) have altered sleep quality, indicating a complex functional relationship between the circadian timing system and adipokine secretion (Laposky et al. 2006). Together, these data suggest that the

circadian timing system regulates daily patterns of lipid metabolism and adipokine secretion. Moreover, rhythmic adipokine feedback may also broadly influence the timing system, a function this hormone shares with circulating melatonin and GCs (Fig. 2.2). Leptin receptors are expressed on SCN neurons and leptin can phase-shift SCN pacemaker neurons in vitro (Prosser and Bergeron 2003). Thus, adipokine secretion, which is regulated by the timing system, has the capacity to modulate the output of the central clock in order to fine-tune control of metabolic homeostasis.

The circadian clock in adipocytes drives endogenous rhythms of lipid metabolism, including free fatty acid (FFA) and TG synthesis (lipogenesis; Johnston et al. 2009). Further, the timing of adipokine secretion is also dependent, in part, on the circadian clock in adipocytes (Brown and Azzi 2013; Otway et al. 2009). Altered clock function in WAT is associated with increased risk of obesity, diabetes, and cardiometabolic disease (Sukumaran et al. 2010; Yoshino and Klein 2013; Johnston 2012). Several gene candidates associated with lipid metabolism are rhythmically expressed under the direct control of the circadian clock in adipocytes (Shostak et al. 2013a, b). These include the genes *atgl* and *hsl*, both rate-limiting lipolytic enzymes whose expression is regulated by the CLOCK:BMAL1 heterodimer through canonical E-boxes (Shostak et al. 2013a). Rhythmic patterns of glycerol release from fat pads in vitro, recording of PER2::luciferase expression rhythms, and qPCR analyses of *atgl* and *hsl* expression in WAT tissue explants confirm autonomous clock function in WAT. Clearly, the molecular clock plays a significant role in the circadian rhythms of both lipid metabolism and hormone and lipid secretion from WAT. The targeting of clock function in adipose as an approach to the treatment of obesity and its affiliated health issues (e.g., risk of T2D) is an area of considerable interest. Small molecules that target REV-ERBα, also a cognate receptor for heme, have potential for the treatment of obesity (Burris 2008; Solt and Burris 2012; Solt et al. 2012; Burris et al. 2012). It has also been determined that exercise and caloric restriction can both synchronize and entrain peripheral clocks, suggesting that the effects of circadian disruption on metabolism may be ameliorated by proper diet and exercise (Schroeder et al. 2012; Hughes and Piggins 2012; Zanquetta et al. 2003; Mendoza et al. 2012). Though certainly promising, these data are limited and have yet to define a mechanistic or causal relationship between normalized timing and improved metabolic homeostasis.

2.10 Summary and Future Perspectives

As we continue to unravel the web of integration between the circadian timing system and endocrine physiology, we grow more and more impressed with the elegant complexity inherent to these deeply entwined physiological systems (see Fig. 2.2). The timing of endocrine rhythms is a pervasive aspect of mammalian physiology that is critical for life on a rotating planet. Though we have made great strides in our understanding of the timing system's role in endocrine physiology, we

have certainly only seen the tip of the proverbial iceberg. The depth of integration, at the cellular and molecular level, remains to be determined for the majority of hormones. It is clear that the timing system plays a significant role in adrenal, pituitary, pineal, and ovarian physiology. The clock also regulates the timing of bone modeling and daily rhythms of glucose and lipid metabolism. While we certainly know a great deal, many critical questions remain. How does the clock regulate the response of peripheral targets to hormones, particularly at the level of each endocrine tissue? Moreover, the role of the clock in each of the many hormone secreting pituitary cells remains largely unknown. How might adjusting the timing of the clock in individual tissues or altering synchrony among peripheral endocrine clocks improve physiological function and/or diminish the risk of endocrine pathophysiology? Is there great promise for treatment of endocrine disorders among the ever-growing library of chronopharmacological agents? As we face a growing epidemic of obesity and diabetes, the information we gain from the study of clock function in these tissues will be the key that unlocks the door to discovery.

Life in a 24-h world continues to present new and exciting challenges for endocrine physiology. The negative influence of rotating shift work, night eating, light-at-night, and sleep loss on metabolism highlights the importance of integration between these two physiological systems. Life against the clock has, and will continue, to be recognized as a significant danger to endocrine physiology, marked by enhanced risk of diabetes, cardiometabolic disease, obesity, and infertility (Fig. 2.3). Though still in their infancy, the fields of chronotherapeutics and chronopharmacology have taken aim at this threat to our health and prosperity. Appreciating the impact of the timing system on mammalian physiology allows us to harness this information in various ways, including enhancing the efficacy of existing therapies. By reducing the duration of exposure to sleep/wake disruption, improving sleep quality, and minimizing the negative influence of light, it may be possible to stem the tide. We can only hope that a more complete understanding of the integration between circadian timing and endocrine physiology will one day lead to the discovery of new and effective pathways for the treatment of endocrine disease.

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Chapter 3
Circadian Regulation of Sleep

Kazuo Mishima

Abstract Both the circadian rhythm that runs biological processes time dependently on a 24-h cycle and the homeostatic mechanism that secures sleep as a measure against fatigue during wakefulness are involved in maintaining a regular sleep–wake cycle. Although the regulatory mechanism of sleep and wakefulness was discussed only conceptually in the early years, the neurophysiological and molecular biological bases underlying sleep and wakefulness have been rapidly elucidated in recent years. The promotion of sleep and maintenance of wakefulness are driven by distinct groups of nerve nuclei that form a negative feedback loop, or a flip-flop circuit, with nerve projections that reciprocally activate and deactivate each other. In addition, the 24-h cycle of sleep and wakefulness is governed by these nuclei due to the neuronal or hormonal input from the suprachiasmatic nucleus, the biological clock.

Keywords Sleep–wake • Circadian • Homeostasis • Neural networks • Two-process model • Thermoregulation • Melatonin • Environmental light

3.1 Neural Mechanism of Sleep and Wakefulness in the Brain

3.1.1 Wake-Promoting Networks

The nuclei and pathways essential for the onset and continuation of the sleep–wake cycle have been comprehensively described by Saper et al. (2001). The tuberomammillary nucleus (TMN) in the posterior hypothalamus, where the histaminergic nerves originate, plays a key role in the maintenance of wakefulness, acting as an arousal center. The histaminergic neurons in the TMN project widely to the entire brain, and activation of this signaling pathway induces brain waves, thereby promoting wakefulness. It is well known that antihistamines (histamine 1 [H1]

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receptor antagonists) induce sleepiness by blocking the histaminergic nerves originating in the TMN. The cholinergic nerves originating in the basal forebrain (BF), laterodorsal tegmental nuclei (LDT), and pedunculopontine tegmental nuclei (PPT) are activated during wakefulness, and sleep is induced by suppressing the activity of these nerves. The cholinergic nerve projections from the LDT and PPT excite the cerebral cortex via the nonspecific nuclei in the thalamus, and this system corresponds to the dorsal (classical) pathway of the ascending reticular activating system (ARAS). On the other hand, the noradrenergic nerve projections from the locus coeruleus (LC) promote wakefulness across a wide area of the brain, by first projecting via the medial forebrain bundle to the Meynert nucleus in the BF and from there to many brain areas via the cholinergic pathways, and this system corresponds to the so-called ventral (alternative) pathway of the ARAS. These two ARAS systems of cholinergic projections involved in wakefulness are clearly distinguished from the histaminergic projections from the TMN. The latter may also enhance wakefulness via the ARAS by projecting to the nonspecific thalamic nuclei (the dorsal pathway), the LDT/PPT, the Meynert nucleus (the ventral pathway), and the LC. Orexin neurons are another group of neurons mediating wakefulness. Orexin was first identified as a neuropeptide that causes narcolepsy, the primary symptom of which is hypersomnia (Chemelli et al. 1999). Orexin neurons, found exclusively in the lateral hypothalamic area (LHA), project to many parts of the brain such as the BF, LDT/PPT, LC, and raphe nucleus (Raphe), which is the origin of serotonergic projections, in addition to the sleep centers described below.

3.1.2 Sleep-Promoting Networks

The ventrolateral preoptic area (VLPO) and medial preoptic area (MnPN) in the anterior hypothalamus play key roles as sleep centers in the onset of sleep (nonrapid eye movement [NREM] sleep). While neural activity in the VLPO neurons specifically increases during sleep, the VLPO also sends inhibitory inputs to the TMN arousal center via gamma-aminobutyric-acid (GABA)-ergic and galanergic projections. In other words, the VLPO is involved in reciprocal functions: the onset of sleep and the suppression of the nucleus in the arousal center. The VLPO sends inhibitory projections to various arousal centers, such as the LC, Raphe, and LDT/PPT, in addition to the TMN. Similar to the VLPO, another preoptic area in the anterior hypothalamus, the MnPN, also possesses GABAergic neurons whose activity increases upon sleep onset, suggesting that the MnPN is part of the sleep center. The MnPN also sends inhibitory projections to various arousal systems such as the cholinergic neurons in the BF, as well as to the LC, Raphe, and LA mentioned earlier. These findings suggest that the balance between sleep and wakefulness is maintained by strong neural connections between the sleep and arousal centers in and around the hypothalamus.

3.1.3 *Timing of Activities of the Sleep- and Arousal-Specific Neurons*

Saper also showed the reciprocal changes in the activities of sleep-specific neurons in POAH (slow wave and paradoxical sleep-specific neurons) and the arousal-specific neurons (in the LC, TMN, and BF) in the transitional state between sleep and wakefulness in mice (Saper et al. 2010). During the transition from NREM sleep to wakefulness, while the activity of sleep-specific neurons in POAH decreases prior to awakening, the neural activity in the arousal-specific neurons in BF and LC increases. On the other hand, the activity of arousal-specific TMN neurons increases after arousal, suggesting that the TMN plays a role in maintaining wakefulness instead of working as the trigger. During the transition from wakefulness to light NREM sleep, the activity of the arousal-specific neurons (LC, TMN, and BF) gradually decreases toward sleep onset, whereas the activity of the sleep-specific neurons increases prior to sleep onset.

Another pathway involves the prostaglandin D2 (PGD2) receptors present in the arachnoid membrane near the BF arousal center. Because the activation of these receptors leads to an increase in free adenosines in the subarachnoid space and because the sleep-inducing action of PGD2 is inhibited by A2 receptor antagonists, the sleep-inducing action of PGD2 is believed to be promoted through the activation of nearby VLPO neurons expressing adenosine A2 receptors by adenosine, which is produced upon the activation of arachnoid PGD2 receptors in the BF. Incidentally, caffeine—a well-known agent of arousal—is an adenosine receptor antagonist.

3.2 Circadian Sleep–Wake Cycle Dependent on the Biological Clock

The biological phenomenon where neural activities in the sleep and arousal centers alternate between increasing and decreasing due to their reciprocal inhibition is often compared to the flip-flop phenomenon in an electrical circuit (Saper et al. 2001, 2010). However, to generate 24-h sleep–wake cycles, circadian signal input is needed from the suprachiasmatic nucleus (SCN)—the biological clock—to the sleep and arousal centers in the VLPO and TMN. SCN nerve projections are involved in various physiological mechanisms including sleep and wakefulness. Strangely, the SCN projects only very few nerves directly to the sleep- and arousal-specific nuclei (Watts et al. 1987). Instead, it has a strong projection to the nearby subparaventricular zone (SPZ) in the hypothalamus. This projection is thought to be essential for the generation of sleep–wake-related circadian signals as sleep–wake cycles disappear almost completely when the SPZ is excised (Lu et al. 2001). However, like the SCN, the SPZ also has very few direct projections to the sleep- or arousal-specific nuclei. Instead, it projects to the dorsomedial

hypothalamic nucleus (DMH) (Chou et al. 2003; Deurveilher and Semba 2005), which contains populations of GABAergic neurons projecting to the VLPO and glutamatergic neurons projecting to the LHA, where the orexin nucleus is (Chou et al. 2003; Thompson et al. 1996). Excision of the DMH results in almost complete disappearance of circadian rhythms in sleep–wake cycles, feeding, and the hypothalamic–pituitary–adrenal system (Chou et al. 2003; Saper et al. 2005), suggesting that the SCN projections to the SPZ and DMH are involved in the generation of circadian sleep–wake cycles.

3.3 Model of Sleep–Wake Regulation

The two-process model proposed by Borbely and Dann is essential to understanding the relationship between the sleep–wake and biological clock systems in humans (Borbely 1982; Daan et al. 1984). This model describes the timing of sleep onset and arousal using two processes: process S and process C.

Sleep is affected by the duration and intensity of preceding wakefulness and the duration of afternoon napping. After prolonged wakefulness due, for example, to staying up all night, there is increased sleep propensity during the day. The duration of wakefulness is also correlated with the amount of slow wave sleep (NREM sleep). The longer the duration of continuous wakefulness before sleep onset (i.e., the duration of sleep deprivation), the greater the amount of slow wave sleep after sleep onset, and the amount of slow wave sleep decreases as the night progresses. In humans, sleep debt caused by prolonged wakefulness is resolved by the homeostatic mechanism called process S (i.e., the sleep process). On the other hand, since sleep is a rhythmic phenomenon that typically appears time dependently in a 24 h cycle, variation in the threshold of sleep and wakefulness controlled by the biological clock is defined as process C (i.e., the circadian process). Process C consists of the circadian fluctuations in sleepiness required to fall asleep (process C) and induce wakefulness (process C). The relationship between sleep propensity and the circadian rhythm of core body temperature is a well-known physiological phenomenon related to process C, as will be discussed later.

According to the two-process model, process S increases monotonically during wakefulness due to the accumulation of sleep substances, and sleep is induced when process S intersects with process C. During sleep, process S decreases exponentially, and when it intersects with process C it induces wakefulness. When there is a period of prolonged wakefulness due, for example, to sleep deprivation, the process S continues to extend and bypasses the intersection with process C. After subsequent sleep onset, sleep continues until it intersects with process C.

This model is characterized by its use of the interaction between the biological clock mechanism and sleep–wake homeostasis to explain the timing of sleep onset and end of sleep. However, the model is also associated with several problems. For example, slow wave sleep, which appears during the latter half of sleep, cannot be explained by the theory that process S accumulates during wakefulness and is

discharged as slow wave sleep during sleep. The revised theory for the model hypothesizes that REM sleep has a role similar to arousal in relation to process S: it suggests that process S accumulates, rather than discharges, during REM sleep, and slow wave sleep therefore appears again in response to an increasing amount of REM sleep during the latter half of sleep.

3.4 Functional Association Between the Circadian and Homeostatic Systems

Previous research findings suggest a functional association between the biological clock and sleep homeostasis. For example, in rats, mice, and squirrel monkeys, the breakdown of the SCN or the functional deletion of clock genes such as *Cry1*, *Cry2*, and *Bmal1* not only abolish the activity-rest rhythm, but also shorten the period of activity (i.e., the period of wakefulness) (Chou et al. 2003; Edgar et al. 1993; Laposky et al. 2005; Wisor et al. 2002). This suggests that the biological clock plays a role in increasing the level of wakefulness itself, presumably by suppressing the neuronal activity of the VLPO through circadian signal input from the SCN via the DMH while also activating the LHA (orexin nerves). Furthermore, compared with evening chronotypes, individuals with the *Per3* polymorphism associated with morningness preference are susceptible to sleep debt and experience strong sleepiness during periods of prolonged wakefulness (Viola et al. 2007). Subsequent clinical studies have also demonstrated association of *Per3* polymorphisms with circadian rhythm sleep disorders (Hida et al. 2014).

3.5 Functional Association Between Sleep and Physiological Rhythms

To maintain high-quality sleep and sufficient daytime wakefulness, it is essential for the sleep–wake cycle and various other physiological rhythms to produce circadian changes with proper phase relationships between them, under the control of the biological clock. To help understand the functional association between sleep and other physiological rhythms described so far, Richardson showed the schematic of sleep regulation by the biological clock and sleep–wake homeostasis (Richardson 2005). The need for sleep is generated as fatigue continues to accumulate during wakefulness. This need rapidly decreases after sleep onset and disappears when individuals get sufficient sleep, which leads to awakening. Circadian rhythms affect the timing of sleep onset and the duration of continuous sleep, which is controlled by the intensity of wake signals in the SCN. Under normal circumstances, the period 2–3 h before sleep onset is known as the prohibited zone because the level of wakefulness is at its highest (Lavie 1986). A certain level of

wakefulness can be maintained even during the latter part of the day because wake signals intensify antagonistically as the need for sleep accumulates during wakefulness. If wake signals did not intensify, it would not be possible to have a good social life beyond the early evening because of the gradually accumulating need for sleep.

Sleepiness intensifies rapidly within a couple of hours after the prohibited zone. In this time zone, various sleep-promoting physiological mechanisms undergo changes in their circadian rhythms—such as an onset of nocturnal melatonin secretion, a decline in core body (and brain) temperature, and an inhibition of glucocorticoid secretion—while maintaining proper phase relationships with each other. The secretion of melatonin is further promoted by the inhibition of melatonin receptor type 1 in the SCN neurons by melatonin secreted from the pineal body, indicating that melatonin and the SCN are in a mutually suppressive relationship. Because the secretion of melatonin rapidly suppresses the activity of the SCN, some researchers even hypothesize that melatonin "opens the sleep gate." Ramelteon (Rozerem®; Takeda Pharmaceutical Co.), which was recently released into the market, is an agonist of melatonin receptor type 1 and promotes sleep onset.

3.6 Sleep and Thermoregulation

As mentioned earlier, the quality and quantity (duration) of sleep correlate closely with various temperature rhythms. Under synchronized conditions, as sleep onset time nears, the body prepares to release heat by increasing skin temperature mainly in the distal arms and legs and subsequently reducing the core temperature. In contrast, as wake time nears, the body prepares to promote thermogenesis by reducing skin temperature and subsequently increasing core temperature. These thermoregulatory responses are not simple physiological phenomena coupled with sleep. Rather, they have deep functional relevance to both sleep homeostasis and circadian rhythms.

Thermoregulation is the most important and fundamental of the body's maintenance systems in homeothermic animals. Exposure to harsh diurnal and seasonal variations in environmental temperature means that homeothermic animals require a large amount of energy to maintain stable thermoregulation. It is therefore quite appropriate that they develop a system to minimize energy consumption during rest or sleep phases. One of the most effective measures to suppress energy consumption is to reduce metabolic rate during sleep. In fact, in mammals, the higher the metabolic rate required to maintain homeothermism, the greater the amount of sleep needed (Zepelin and Rechtschaffen 1974).

Thermoregulation has not been fully elucidated, but the mutual balancing between the warm and cold sensitive neurons in the preoptic/anterior hypothalamus (POAH), which is based on the neural and hormonal information from the skin and body core, is believed to drive the core thermoregulatory system. According to the

most widely accepted servo-control model, upon receiving circadian signals from the SCN, the POAH warm and cold sensitive neurons modify temperature sensitivity thresholds to change the balance between heat production and release in order to approach the set-point for the core temperature for that time of the day. In humans, it is speculated that the core temperature can be adjusted quickly by regulating the balance between heat production and loss to meet the set-point changing continuously in a time-dependent manner. Indeed, the SCN is known to send various nerve projections to the POAH and other nuclei involved in sleep–wake regulation [see the review by Van Someren et al. (2002)]. Moreover, animal studies have revealed that sleep is induced by physically warming (McGinty and Szymusiak 1990; Gong et al. 2000) or pharmacologically stimulating (Ramesh et al. 1995) the POAH. The activity of the POAH warm sensitive neurons was also shown to increase at sleep onset and decrease immediately before waking (Alam et al. 1996; Sherin et al. 1996). Furthermore, the activation of the POAH warm sensitive neurons is needed to regulate the activity of various nuclei involved in the sleep–wake cycle, such as the posterior hypothalamus, BF, and dorsal Raphe (Szymusiak 1995; Guzman-Marin et al. 2000). The above findings strongly indicate that the thermoregulatory mechanism via the POAH plays a key role in the onset and maintenance of sleep.

Heat is generated mainly by the shivering of skeletal muscles and by brown adipocytes, while heat is dissipated by adjusting skin temperature through sweating, breathing, and expanding peripheral blood vessels. In humans, heat production and metabolic rate are reduced by decreasing the set-point of body temperature in synchronization with the onset of NREM sleep, expansion of the peripheral blood vessels, elevation of skin temperature, increased sweat production, and a drop in core temperature. During REM sleep, on the other hand, these thermoregulatory responses to adjust the set-point are inhibited. The major mechanism of thermoregulation during human REM sleep, in which skeletal muscle atonia occurs, is the suppression of heat loss and a marked reduction in sweating. The metabolic responses of POAH toward thermal stimuli also disappear during REM sleep in animals (Glotzbach and Heller 1976). Single-neuron recordings of POAH warm and cold sensitive neurons have shown that their firing rate changes in line with changes in POAH temperature during NREM sleep but not during REM sleep (Parmeggiani 1985), suggesting that the disappearance of thermoregulatory and metabolic responses during sleep is due to inhibition of the thermal sensitivity of the POAH neurons.

3.7 Circadian Body Temperature Rhythms and Sleep

As described earlier, sleep and core temperature maintain a certain phase relationship under synchronized conditions. Sleep begins during the downward phase of circadian temperature rhythm, whereas wakefulness begins during its upward phase immediately after the body temperature reaches its nadir. Even when internal

desynchronization occurs in an isolated environment (Wever 1979), the sleep–wake cycle and circadian temperature rhythm maintain a functional relationship, instead of running freely with different circadian rhythms. At a glance, this mutual phase relationship appears to change irregularly during internal desynchronization, but analysis of the correlation between voluntary bedtime or spontaneous sleep onset time and phase of circadian temperature rhythm has shown that sleep onset occurs most frequently around the time of the core temperature nadir, while waking occurs most frequently around the core temperature peak (Czeisler et al. 1980a; Zulley et al. 1981). The duration of sleep becomes shortest or longest as sleep onset time nears the nadir or peak of body temperature, respectively. This indicates that the duration of continuous sleep is greatly influenced by the phase of circadian temperature rhythm at sleep onset. As a result, when free running, the duration of sleep changes periodically along with changes in the phase relationship between the sleep–wake cycle and circadian temperature rhythm.

Sleep not only forms a 24-h rhythm with wakefulness in a complementary manner, but also generates REM–NREM sleep cycles as a periodic internal phenomenon. Under synchronization conditions, NREM (slow wave) sleep appears mainly in the first half of the night, and REM sleep increases during the second half of the night. Unlike slow wave sleep that depends greatly on wakefulness before sleep onset, the onset of REM sleep is closely related to circadian temperature rhythm. A study investigating subjects who repeated an ultra-short schedule of 2-h sleep and 2-h wakefulness in a supine position in isolation revealed that REM sleep propensity (e.g., the shortening of REM latency) and REM sleep duration are maximized near the core temperature nadir (Czeisler et al. 1980b). Under internal desynchronization, REM sleep appears immediately after sleep onset (sleep-onset REM) due to the intense coupling of REM sleep and core temperature; its sleep architecture is therefore different from that observed under synchronized conditions.

3.8 Biological Clock and Sleep: Involvement in Aging and Disease

3.8.1 Thermal Control, Sleep, and Aging

The close association between thermoregulation and the sleep–wake cycle was described earlier. In addition to the influence of the biological clock, a recumbent position, lights out, mental relaxation, and other behavioral maneuvers before sleep can redistribute blood flow in the body, improving peripheral circulation and promoting heat loss. A study revealed that among many heat-releasing mechanisms, dilation of peripheral capillaries had a particularly strong correlation with the degree of sleep promotion (Krauchi et al. 1999). Sleep-promoting agents such as sleeping pills and melatonin, bathing, calorie intake, phase displacement in the

biological clock, and other actions that promote sleep onset are involved in heat loss (Echizenya et al. 2003, 2004; Horne and Staff 1983; Horne and Shackell 1987; Gilbert et al. 1999). In contrast, when heat loss is blocked, the onset and maintenance of sleep are disturbed. For example, sleep disorders are common in patients with primary vasospastic syndrome, where the mechanism of heat loss is disturbed by the occurrence of peripheral vasospasm (Pache et al. 2001).

In most elderly individuals, the amplitude of the circadian temperature cycle reduces due to nighttime inhibition of heat loss. This appears to be caused by the inhibition of heat loss due to age-related changes in the biological clock system, functional decline in the parasympathetic nervous system, peripheral circulatory disturbances, and atherosclerosis. Functional decline in the heat loss mechanism may also play a role in the characteristic reduction of slow wave sleep in elderly individuals. Benzodiazepine (BZ), the standard drug for the treatment of insomnia worldwide, also modifies thermoregulation. When administered, BZ quickly lowers the core body (brain) temperature by dissipating heat. The dilation of blood capillaries by BZ is an effective predictor of not only hypnotic effects, but also impaired performance in higher brain functions (Echizenya et al. 2003, 2004; Gilbert et al. 1999).

3.8.2 Neuroendocrine Function, Sleep, and Aging

Most hormones show clear circadian variation in their secretion levels and maintain a steady phase relationship with the sleep–wake cycle. While some hormones' secretion patterns are affected by sleep itself, others are more strongly regulated by the biological clock. Representative hormones in the former group are growth hormones and prolactin. Adrenocorticotropic hormone, cortisol, and melatonin belong to the latter group and are regulated directly by the SCN without being affected by a shift in sleep hours or sleep deprivation. Thyroid-stimulating hormone, luteinizing hormone, and testosterone, however, are affected by both sleep and the circadian clock. By maintaining a proper phase relationship with the sleep–wake cycle, these hormones effectively exercise their influence on biological mechanisms including the onset and maintenance of the sleep–wake cycle. For example, because glucocorticoid, which plays a role in gluconeogenesis, anabolic reaction, and the suppression of excessive immune responses, also has an arousing action, it is strongly suppressed during nighttime sleep. The secretion of glucocorticoid increases in the form of a pulse before waking and reaches a peak during the period between wake time and noon when people are active. However, sleep is disturbed due to age-related changes in neuroendocrine dynamics. In elderly individuals, the blood concentration of glucocorticoid increases due to reduced suppression of its nighttime secretion. Moreover, because sensitivity toward glucocorticoid increases with age, elderly individuals are especially susceptible to waking after intravenous administration of glucocorticoid. The lack of the nighttime suppression of glucocorticoid secretion becomes even more pronounced in

patients with vascular dementia or Alzheimer's disease with sleep disturbance (Mishima et al. 2000). The nighttime glucocorticoid secretion is further increased by wakefulness due to insomnia, nighttime activities, and stress. Although age-related decline in hippocampal function is thought to be a causal factor of excessive glucocorticoid secretion (because glucocorticoid potently disrupts hippocampal neuronal activity), together they form a vicious cycle.

3.8.3 Melatonin, Sleep, and Aging

The secretion of melatonin decreases with age, but the exact mechanism behind this remains unclear. Functional alteration of the biological clock and calcification of the pineal body appear to be involved, but poor lighting should be considered as well, because environmental illumination is the most powerful modulator of the human biological clock system. With the exception of special conditions and environments such as total blindness, isolated environments, the Earth's polar regions, and spaceflight, we tend to think that we have enough environmental illumination to maintain our daily lives. However, this perception is not necessarily correct, at least for some elderly individuals. Admission to a care facility or having a physical handicap or dementia may mean that some elderly individuals have significantly fewer opportunities to be exposed to high-intensity natural light and consequently have low levels of melatonin secretion (Mishima et al. 2001; Haimov et al. 1994). However, when elderly individuals who have been placed in low illumination environments receive complementary light exposure with, for example, artificial lighting, their secretion of melatonin increases considerably to a level almost equivalent to that of healthy young controls (recovery), bypassing the level secreted in healthy elderly controls (Mishima et al. 2001). This suggests that a decrease in melatonin secretion in elderly individuals, which is conventionally described as an age-related physiological reaction and irreversible aging process, is in fact caused secondarily by decreased exposure to natural light (high-intensity light) due to the lack of opportunity to go outdoors. It is therefore important to keep in mind that deterioration of the light environment in daily living is an important hindrance to maintaining the sleep–wake and biological clock systems in some elderly individuals who have few opportunities for social contact or daytime activities or who have a decline in sense organ function.

3.8.4 Sleep, Metabolism, and Disease States

Circadian sleep disruption, such as that associated with shift work, correlates with many disease states, including cancer, diabetes, hypertension, and cardiovascular disease [reviewed in Machado and Koike (2014) and Kim et al. (2015)]. This may be related to circadian disruption in levels of hormones such as cortisol and

melatonin (see Chap. 2 on the clock in the endocrine system). Indeed, circadian misalignment and sleep disruption have been associated with endocrine and metabolic disruption, affecting glucose and insulin signaling with consequences such as obesity and decreased longevity [see Morris et al. (2012) for an excellent review]. Much remains to be learned about the connections between sleep, the circadian clock, and metabolic function, but developing work suggests that small molecule-mediated modulation of circadian amplitudes may be beneficial for both sleep quality and metabolic function (Nohara et al. 2015).

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Chapter 4
The Human SCN in Health and Neuropsychiatric Disorders: Postmortem Observations

Ai-Min Bao and Dick F. Swaab

Abstract The suprachiasmatic nucleus (SCN) is the master clock of the mammalian brain. Lesions in the human SCN region due to suprasellar pituitary tumors or metastasis result in a decreased expression of a major SCN peptide, arginine vasopressin (AVP), and in disturbed circadian rhythms. In Nissl-stained paraffin sections, the human SCN cannot be recognized without immunocytochemistry for AVP, vasoactive intestinal polypeptide (VIP), or neurotensin. Gamma-aminobutyric acid is co-localized with one or more peptides in SCN neurons. Compared to monkeys and other animals, the human SCN has very large populations of neurotensin cells and of neuropeptide Y neurons, which obscure a geniculo-hypothalamic tract containing the same peptide. In postmortem human SCN, distinct day–night and seasonal fluctuations were found for the AVP- and VIP-expressing neurons in subjects up to 50 years of age. Moreover, structural and functional differences in SCN are attributed to gender, sexual orientation, and sex differences in aging. The SCN drives circadian and circannual rhythms in the pineal gland production of melatonin that affects many brain functions mediated by the melatonin receptors (MT1 and MT2). Like the SCN, the pineal system also shows strong changes with aging. The retino-hypothalamic tract (RHT) directly innervates the VIP and neurotensin neurons in the SCN and mediates the entraining effects of light on the SCN. The RHT is made up of retinal ganglion cells, which contain pituitary adenylate cyclase-activating polypeptide (PACAP) and co-store glutamate. Light activates these cells via the photopigment melanopsin in the PACAP

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cells. In addition, serotonin and histamine innervate the SCN, while melatonin acting on the SCN is mediated by MT receptors. Immunocytochemical observations show that AVP and VIP fibers innervate the SCN itself, including the contralateral SCN and a number of other hypothalamic areas, including the sub-paraventricular zone and the dorsomedial nucleus. These observations match very well with the results of postmortem tracing following an injection into the human SCN. The strong projection from the SCN to the supraoptic nucleus seen in rodents was not found in human.

The SCN of Alzheimer (AD) patients—who tend to suffer from circadian rhythm disturbances—exhibits a dramatic loss of AVP-, neurotensin-, and MT1-expressing neurons. AVP-mRNA levels decrease in the SCN as early as the preclinical stages (Braak stages I–II), while day–night fluctuations in pineal melatonin production are already disrupted in the preclinical AD stages (Braak II–III). A combination of light and melatonin treatment was found to increase sleep efficiency and improve nocturnal restlessness, mood, performance, daytime energy, and quality of life.

In Huntington's disease patients, the SCN contains 85 % less VIP-expressing neurons and 33 % less AVP-expressing neurons. However, the total amount of VIP- or AVP-mRNA was unchanged. In depression an increased number of AVP-expressing neurons were observed, together with a decreased amount and a diminished circadian fluctuation of AVP-mRNA in the SCN. The number of MT1 was also increased in the SCN in depression, while the number of MT2-expressing cells did not change. In primary hypertension the number of AVP-, VIP-, and neurotensin-containing neurons was reduced by more than 50 %. The amount of AVP-mRNA expression in the SCN in glucocorticoid-exposed patients was found to be two times lower. In addition, there was a 53 % decrease in the total number of profiles in the SCN that expressed AVP-mRNA.

Keywords Suprachiasmatic nucleus • Biology rhythms • Aging • Sexual differentiation • Depression • Alzheimer's disease • Huntington's disease • Hypertension • Glucocorticoids

4.1 Human Suprachiasmatic Nucleus in Health

The suprachiasmatic nucleus (SCN), a structure of only 0.25 mm^3, is located in the hypothalamus, on both sides of the midline on top of the optic nerve, and borders the third ventricle (for microscopic anatomy, see Fig. 4.1). The SCN is the master clock of the mammalian brain and coordinates all hormonal and behavioral circadian and circannual rhythms (Swaab 2003). Lesions in the SCN region, e.g., as a result of suprasellar pituitary tumors or metastasis (Fig. 4.2), were found to result in a decreased expression of one of the major SCN peptides, arginine vasopressin (AVP), and in disturbed circadian rhythms (Swaab 2003; Borgers et al. 2011, 2013).

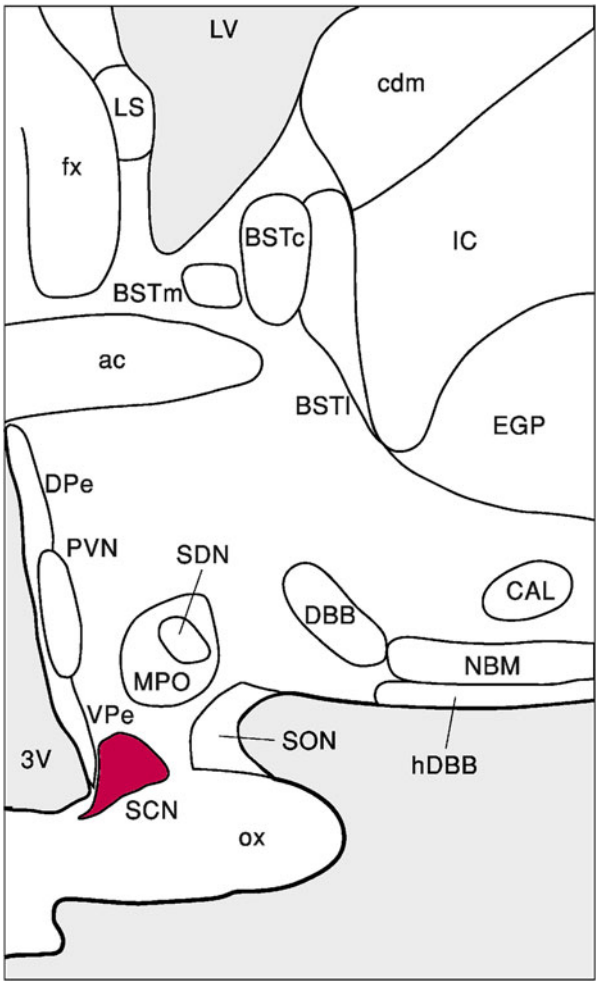


Fig. 4.1 Schematic representation of the nuclei of the human hypothalamus. *Ox* optic chiasm, *NBM* nucleus basalis of Meynert, *hDBB* horizontal limb of the diagonal band of Broca, *SDN* sexually dimorphic nucleus of the preoptic area, *SCN* suprachiasmatic nucleus, *BST* bed nucleus of the stria terminalis (*c* centralis, *m* medialis, *l* lateralis, *p* posterior), *PVN* paraventricular nucleus, *SON* supraoptic nucleus, *DPe* periventricular nucleus dorsal zone, *VPe* periventricular nucleus ventral zone, *fx* fornix, *3V* third ventricle, *ac* anterior commissure [From Swaab (2003); Fig. 4.0, with permission]

4.1.1 Chemoarchitecture

The human SCN cannot be recognized with certainty in the conventional 6–10 μm Nissl-stained paraffin sections (Mai et al. 1991), and immunocytochemical labeling of this structure (i.e., with anti-AVP, anti-vasoactive intestinal polypeptide (VIP) or anti-neurotensin) is thus necessary (for references, see Swaab (2003); Fig. 4.3). By means of immunocytochemistry Mai et al. distinguished five major subdivisions in the human SCN (Mai et al. 1991). The region of the SCN that receives retinohypothalamic tract (RHT) input—and is therefore considered to be important for entrainment—is characterized by VIP neurons (Moore 1992). AVP is found in the remainder of the SCN and neurotensin is found throughout the entire SCN (Moore 1992). In addition, somatostatin, thyrotropin-releasing hormone, galanin

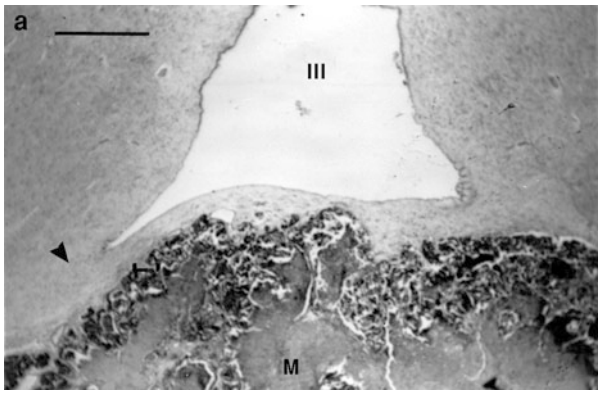


Fig. 4.2 Metastasis affecting function of the suprachiasmatic nucleus. Scully et al. (1983) and Schwartz et al. (1986) have described a 55-year-old postmenopausal female patient with a discrete metastasis of an adenocarcinoma of the rectum in the ventral hypothalamus, optic chiasm, and neurohypophysis (*a*, thionine staining) who, while she was admitted to hospital for the final time, developed an abnormal daily rhythm of oral temperature. She had hypothalamic diabetes insipidus, low FSH, blurring of vision in the periphery of the right temporal field, and required more sleep at night. The metastasis was located between the infundibulum, carotid artery, and optic nerve. The infundibulum was pushed into the hypothalamus. The mass also infiltrated downward along the pituitary stalk. The white granular mass extended into the supraoptic recess. The fornices were pushed laterally by the tumor. We determined 1964 vasopressin-expressing neurons in the SCN, which was only 23 % of the control values for the group of 50- to 80-year-old women (8370 ± 950 vasopressin neurons, $n = 8$). This observation supports the importance of the activity of vasopressin neurons for the expression of circadian rhythms in the human ($Bar = 1$ mm) [From Swaab (2003); Fig. 4.2, with permission]

preproenkephalin, delta-sleep-inducing peptide, leptin receptor, and hypocretin fibers are present in the SCN (Couce et al. 1997; Swaab 2003; Garcia-Falgueras et al. 2011). Furthermore, VIP binding sites, estrogen receptor (ER) α and β, the progesterone receptor (Kruijver and Swaab 2002) (Fig. 4.4), and melatonin receptors (MT) (Wu et al. 2013) were found in the human SCN. Because VIP is present in the SCN, it is not surprising that peptide methionine amide (a peptide with histidine and methionine, PHM) is also present in the human SCN. PHM and VIP are encoded on two adjacent exons of a common prepro-VIP gene. Using confocal laser scanning microscopy, Romijn et al. (1999) found that a small percentage of the neurons in the human SCN co-localized with AVP and VIP.

Following antigen retrieval by microwave treatment of sections, the staining of AVP and VIP becomes more sensitive. Due to this treatment, the volume of the AVP-SCN subnucleus increased 2.4 times and that of the VIP-SCN subnucleus by 4 times; the number of AVP-stained neurons increased by 70 % and the number of VIP-stained neurons increased by 280 %. The neurons that were visible without microwave treatment were localized mainly in the central part of the SCN, whereas the neurons that became visible only after microwave treatment were localized in the peripheral area of the SCN. This suggests that the AVP and VIP neurons in the central part of the SCN contain more peptide, possibly because they are more active

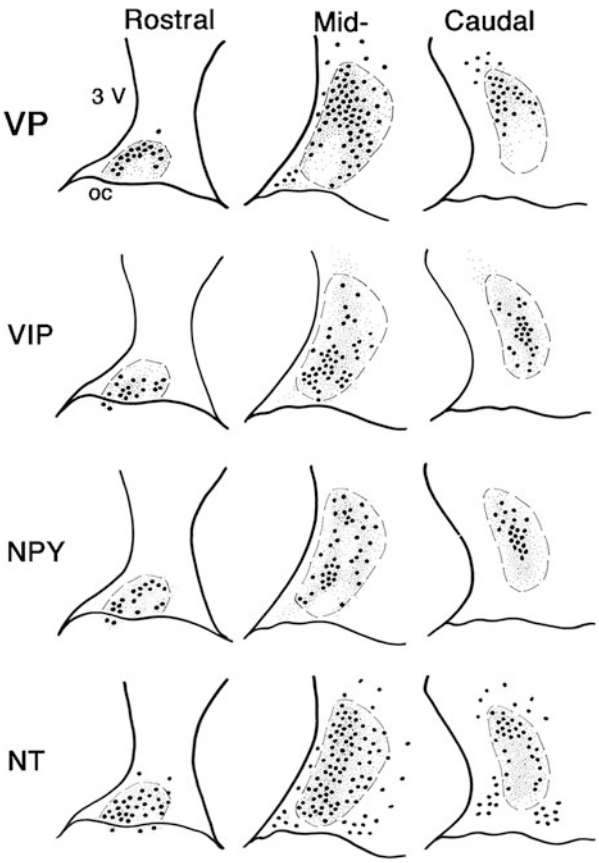


Fig. 4.3 Diagram showing the organization of the human SCN. The distribution of vasopressin (VP), vasoactive intestinal polypeptide (VIP), neuropeptide-Y (NPY), neurotensin (NT), neurons (*large black dots*), fibers (*small gray points*) is shown at three levels, from rostral to caudal [From Moore (1992); Fig. 6, with permission]

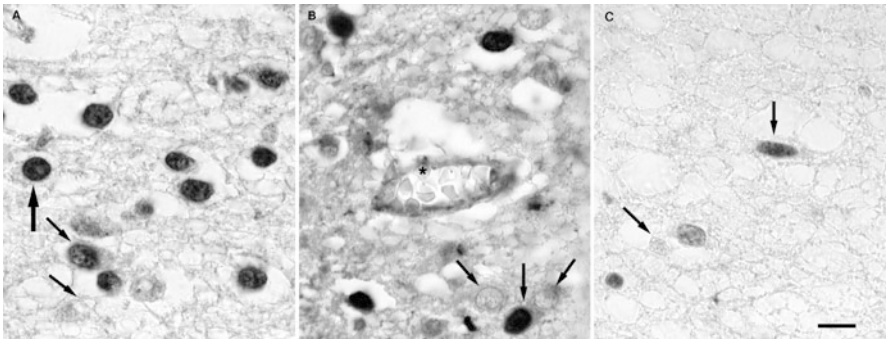


Fig. 4.4 (**a**) Estrogen receptor (ER) α, (**b**) ERβ, and (**c**) progesterone receptor (PR) immunoreactivity in SCN neurons. The *asterisk* points to nuclear ERβ immunoreactivity in smooth muscles and endothelial cells of a small blood vessel. Note the positive and negative nuclear ERα, ERβ, and PR staining in adjacent SCN neurons, as indicated by the *arrows* in (**a**), (**b**), and (**c**). *Scale bar* represents 8 mm [From Kruijver and Swaab (2002); Fig. 3, with permission]

than the peripheral ones (Zhou et al. 1996). Could this be a general characteristic of hypothalamic nuclei?

Many neurons in the human SCN contain the two isoforms of glutamic acid decarboxylase (GAD), GAD65 and GAD67. Gamma-aminobutyric acid (GABA) is co-localized with one or more peptides in SCN neurons (Swaab 2003). GABA is generally known as an inhibitory neurotransmitter in the brain, but its action may depend on the circadian time. SCN neurons can be *excited* by GABA through a $GABA_A$-dependent mechanism. An intermediate density of benzodiazepine binding sites is already present in the SCN of the human fetus and neonate. A dense catecholaminergic network is found in the SCN of the human fetus from as early as the third and fourth months of pregnancy (Swaab 2003).

4.1.2 Day/Night Rhythm

Much of the variability observed in the human SCN is related to its function as the biological clock. In postmortem tissue of a group of young subjects (6–47 years of age), a distinct day–night fluctuation with an asymmetrical, bimodal waveform was found for the AVP- and VIP-expressing neurons in the human SCN (Fig. 4.5) (Hofman and Swaab 1993; Hofman 2000, 2003). The AVP cycle has a peak in the early morning, a lower plateau during the day, a second peak in the late afternoon, and a decline beginning in the early evening, leading to a nadir around midnight. The VIP cycle shows a peak in the middle of the night, a lower plateau beginning in the late night and lasting for about 12 h, and a second peak in the late afternoon, followed by a sharp decline in the early evening (Hofman 2003). Time series analysis showed that the circadian cycles in the human SCN can be adequately described by a model consisting of nonlinear periodic functions, which could be decomposed into monophasic and diphasic cycles, with periods of 24 and 12 h, respectively (Hofman 2003). The demonstration of these two significantly different, but temporally linked, output profiles suggests that the SCN contains more than one oscillator. Circadian rhythms are also present in the human retina (Tuunainen et al. 2001), which may be the consequence of circadian rhythms in the SCN, or it could be based upon intrinsic circadian oscillations, and, through the RHT, influence the rhythms in the SCN.

Interestingly, since the biological clock stops at the time of death, it is possible to read the time of death in forensic tissues by measuring gene expression levels of various clock genes and their ratios (Kimura et al. 2011).

4.1.3 Seasonal Rhythm

Strong seasonal fluctuations were also observed in postmortem human SCN tissue. The number of AVP- and VIP-containing neurons in the SCN was found to change

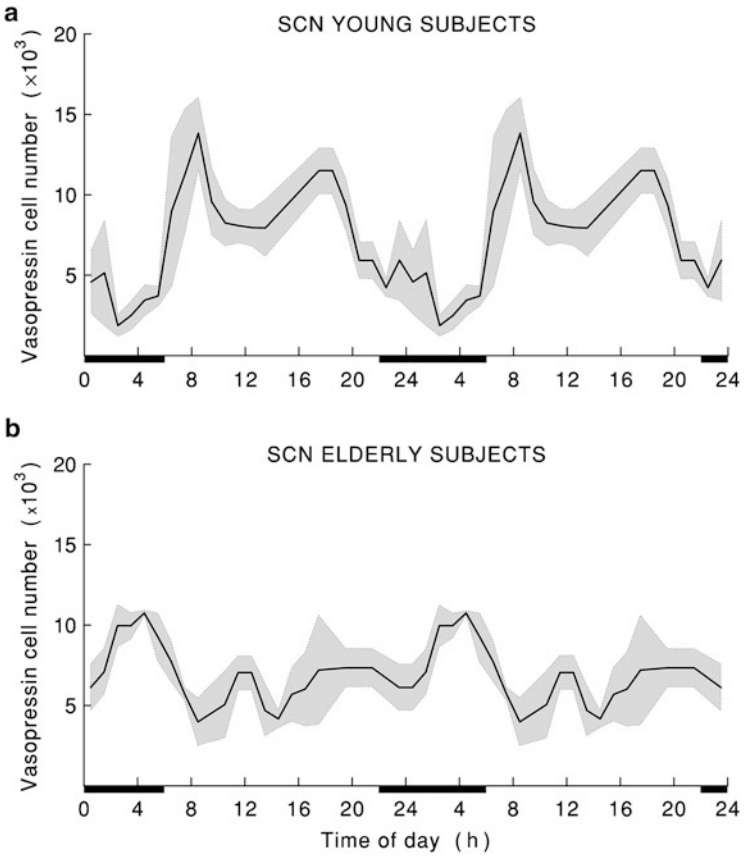


Fig. 4.5 Circadian rhythm in the number of vasopressin-containing neurons in the human suprachiasmatic nucleus (SCN) of (**a**) young subjects (<50 years of age) and (**b**) elderly subjects (>50 years of age). The *black bars* indicate the night period (22:00–06:00 h). The general trend in the data is enhanced by using a *smoothed double-plotted curve* and is represented by mean ± S.E. M. values. Note the circadian rhythm in the SCN of young people with low values during the night period and peak values during the early morning [From Hofman and Swaab (1994); Fig. 1, with permission]

in the course of a year, with August–September values being two times higher than April–May values (Fig. 4.6) (Hofman and Swaab 1992b; Hofman 2004). Photoperiod seems to be the major Zeitgeber (German for "time-giver" or pacemaker) for the observed annual variations in the SCN (Hofman and Swaab 1993).

The hypothalamic levels of serotonin and dopamine, neurotransmitters known to innervate the SCN, show diurnal rhythms and seasonal rhythms as well (Carlsson et al. 1980). In addition, reduced thalamus/hypothalamus serotonin transporter availability was observed in living patients by single-photon emission computed tomography in winter compared with summer, at least for female subjects (Neumeister et al. 2000). It remains to be determined exactly how these seasonal

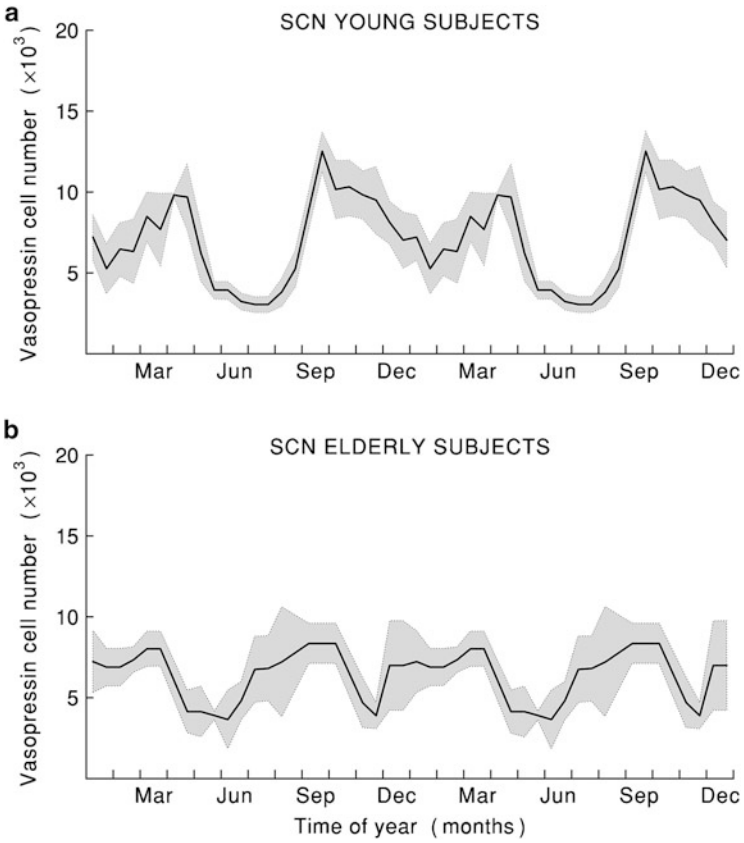


Fig. 4.6 Annual rhythm in the number of vasopressin-containing neurons in the human suprachiasmatic nucleus (SCN) of (**a**) young subjects (<50 years of age) and (**b**) elderly subjects (>50 years of age). The general trend in the data is enhanced by using a *smoothed*, *double-plotted curve* and is represented by mean ± S.E.M. Note the circannual rhythm in the SCN of young people with low values during the summer and peak values in the autumn period [From Hofman and Swaab (1995); Fig. 1, with permission]

fluctuations causally relate to the SCN circannual rhythms. However, the fact that both serotonin and dopamine circadian and seasonal rhythms are observed in the hypothalamus suggests that the SCN drives the monoaminergic systems instead of the other way around. Furthermore, a notable seasonal variation in the volume of the hypothalamic paraventricular nucleus (PVN) was observed in postmortem human brain material. The PVN volume reached its peak during the spring (Hofman and Swaab 1992a).

4.1.4 Pineal Gland

An important component in the circadian and circannual timing system is the pineal gland. Melatonin production varies with age, time of the day, and season. The nocturnal excretion of the major melatonin metabolite, 6-sulfatoxymelatonin, was three times higher in healthy full-term infants of 8 weeks of age, born in summer, than that of those born in winter. When exposed to long nights, the duration of melatonin and prolactin secretion and the rise in cortisol are longer than the duration of secretion found during short nights. The seasonal variations were no longer present at 16 weeks of postnatal age, suggesting a prenatal influence of the photoperiod on the ontogeny of melatonin (Sivan et al. 2001). There is a relationship between diurnal and seasonal pineal rhythms. The diurnal rhythms in pineal melatonin content of autopsy material are evident only in the long photoperiod (i.e., April–September), with melatonin concentrations being four times higher at night (22.00–10.00 h) than during the day (10.00–22.00 h) (Hofman et al. 1995). This seasonal effect was confirmed by Luboshitzky et al. (1998). In contrast, diurnal variations in the pineal 5-methoxytryptophol contents are only observed in the short photoperiod (i.e., October–March) with high concentrations during the day and low concentrations at night (Hofman et al. 1995). This shows that the synthesis of indolamines in the human pineal exhibits a diurnal rhythm that is affected by seasonal changes in day length (Hofman et al. 1995). Seasonal rhythms are also found in gonadotropin receptors in the pineal gland, with higher values in the winter than in the summer (Luboshitzky et al. 1997).

Melatonin is implicated in numerous physiological processes, including circadian rhythms, stress, and reproduction, many of which are mediated by the MTs. Immunocytochemistry was used to study the distribution of the MT1 in the postmortem human hypothalamus and pituitary. In addition to its localization in the SCN, a number of novel sites, including the PVN, periventricular nucleus, supraoptic nucleus (SON), sexually dimorphic nucleus, the diagonal band of Broca, the nucleus basalis of Meynert, infundibular nucleus, ventromedial and dorsomedial nucleus, tuberomamillary nucleus, mamillary body, and paraventricular thalamic nucleus, were observed to have neuronal MT1 receptor expression. Moreover, the MT1 receptor was co-localized with some AVP neurons in the SCN, co-localized with some parvocellular and magnocellular AVP and oxytocin neurons in the PVN and SON, and co-localized with some parvocellular corticotropin-releasing hormone (CRH) neurons in the PVN. In the pituitary, strong MT1 expression was observed in the pars tuberalis, while a weak staining was found in the posterior and anterior pituitary. The co-localization of MT1 and CRH suggests that melatonin might directly modulate the hypothalamic–pituitary–adrenal (HPA) axis in the PVN, which may have implications for stress conditions such as depression (Wu et al. 2006b, 2013).

4.1.5 Sexual Differentiation in the SCN

Structural and functional sex differences are present in the SCN [for a review, see Bailey and Silver (2013)]. Staining with anti-AVP showed that the shape of the human SCN is sexually dimorphic, i.e., more elongated in women and more spherical in men, but the AVP cell number and the volume of the SCN-AVP subnucleus are similar in both sexes (Swaab et al. 1985). These gender differences and the presence of sex hormone receptors (ER and PR; see Fig. 4.4) (Kruijver and Swaab 2002) in the SCN indicated the possible involvement of this nucleus in sexual behavior and reproduction (Swaab 2003; Bailey and Silver 2013). In addition, sex differences are present in sleep, i.e., a shorter average intrinsic circadian period was observed in women, which may have implications for understanding the sex differences in habitual sleep duration and insomnia prevalence (Duffy et al. 2011).

Moreover, SCN structure has a link with sexual orientation. Morphometric analysis of the human hypothalamus revealed that the volume of the SCN in homosexual men is 1.7 times larger as that of a reference group of male subjects and contains 2.1 times as many cells, while another hypothalamic nucleus, located in the immediate vicinity of the SCN, the sexually dimorphic nucleus (SDN), showed no such differences in either volume or cell number (Fig. 4.7) (Swaab and Hofman 1990). The SDN data indicate the selectivity of the enlarged SCN in homosexual men, but do not support the hypothesis that homosexual men simply have a "female hypothalamus" (Swaab and Hofman 1990, 1995).

In humans, during normal aging, the number of peptidergic neurons in the SCN deteriorates in a sexually dimorphic way. The number of VIP-expressing neurons in the SCN of women does not change during aging, whereas in men a complex pattern of changes is observed. Between 10 and 40 years of age, the male SCN contains twice as many VIP neurons as the female SCN, but a subsequent decrease in the number of male VIP neurons between 40 and 65 years of age results in fewer VIP neurons in men than in women. After 65 years of age, the sex difference remained just short of significance (Fig. 4.8) (Zhou et al. 1995).

4.1.6 Aging

With advancing age, the circadian timing system in humans is progressively disturbed as is clearly demonstrated by a reduced amplitude and period length of circadian rhythms and an increased tendency toward internal desynchronization [for reviews, see Van Someren (2000)].

There are indeed signs of neuronal degeneration of the SCN during aging. In the human SCN, the number of AVP-expressing neurons exhibits a marked diurnal oscillation in young (up to 50 years of age), but not in elderly people (over 50 years of age) (Hofman and Swaab 1994). Whereas in young subjects, low

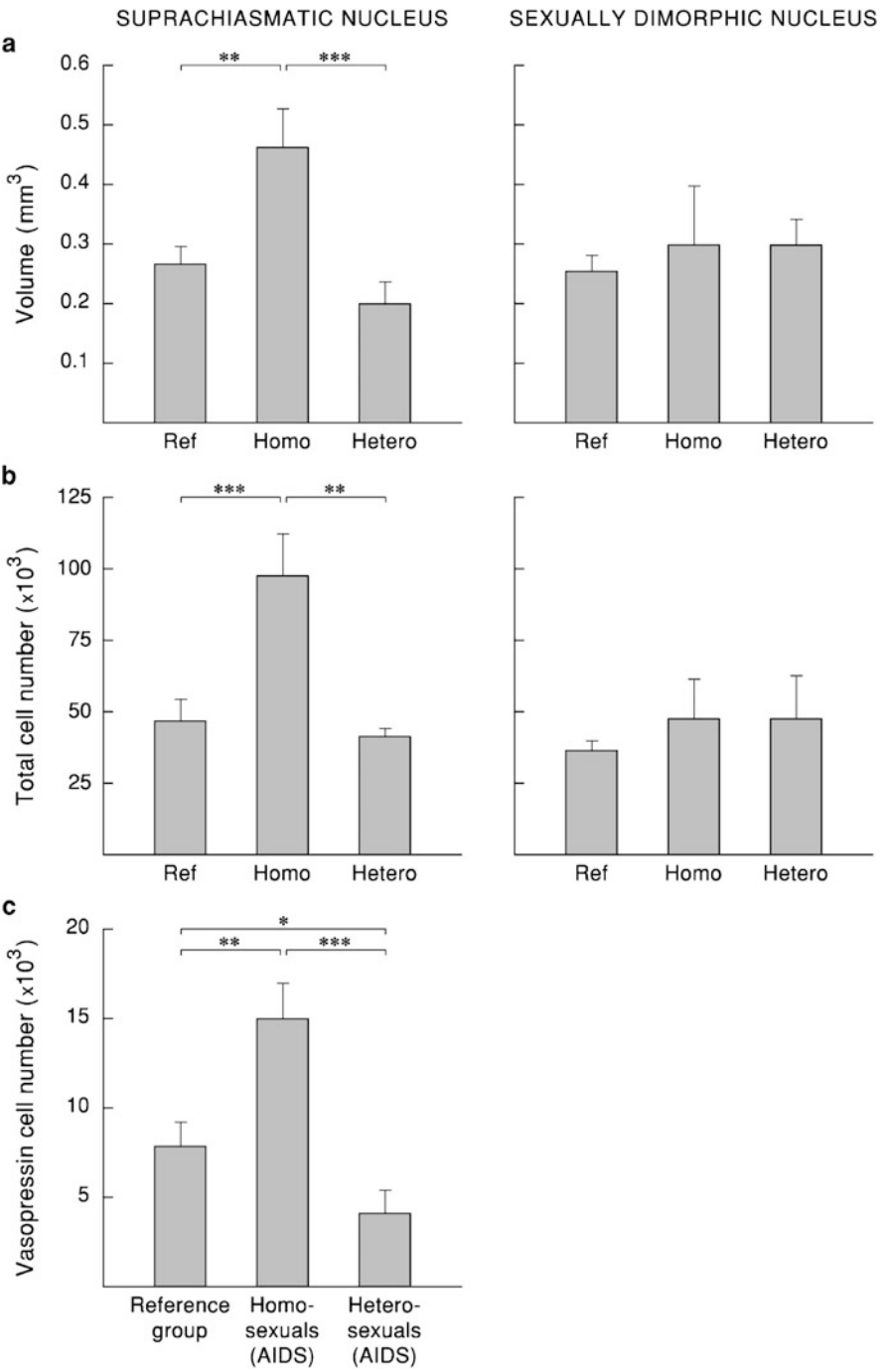


Fig. 4.7 (**a**) Volume of the human suprachiasmatic nucleus (SCN) and sexually dimorphic nucleus of the preoptic area (SDN) as measured in three groups of adult subjects: (1) a male reference group ($n = 18$); (2) male homosexuals who died of AIDS ($n = 10$); (3) heterosexuals who

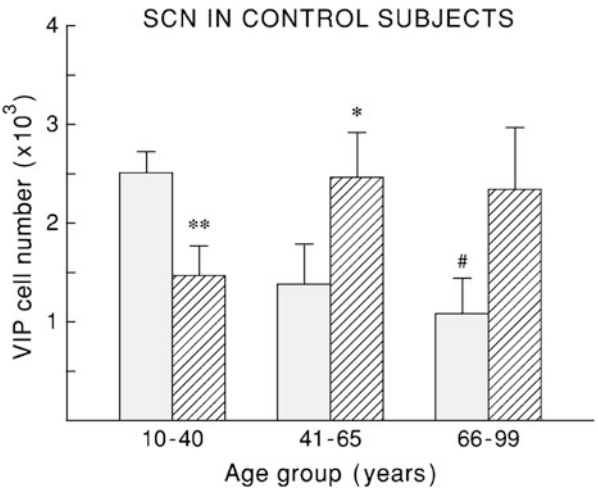


Fig. 4.8 Lifespan changes in the number of vasoactive intestinal polypeptide (VIP)-immunoreactive neurons of the human SCN in control subjects. The *blank bar* indicates the males and the *hatched bar* indicates the females. The SCN of young males (10–40 years) contains twice as many neurons as that of young females ($^{**}p < 0.02$). This sex difference reverses in middle-aged subjects ($^{*}p < 0.04$). Note that the decrease in the number of VIP cells in males already begins in middle age and there is a significant reduction in the elderly males compared with young males ($\#p < 0.02$) [From Zhou et al. (1995); Fig. 2, with permission]

AVP-expressing neuron numbers were observed during the night and peak values during the early morning, a disrupted cycle with reduced amplitude was found in the SCN of elderly people. Similar age-related decrements have been reported for the seasonal timing system (see Fig. 4.6) (Hofman and Swaab 1995). As the fluctuating number of AVP-expressing neurons probably reflects the peptidergic activity state of the cells, these findings suggest that the temporal organization of these neuronal rhythms becomes progressively disturbed in senescence. It is of interest to notice that more frequent and prolonged awakenings and shorter sleep

Fig. 4.7 (continued) died of AIDS ($n = 6$; 4 males and 2 females). The values indicate medians and the standard deviation of the median. The differences in the volume of the SCN between homosexuals and the subjects from both other groups are statistically significant (Kruskal–Wallis multiple comparison test, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Note that none of the parameters measured in the SDN (A, B) showed significant differences among the three groups (p always > 0.4). (**b**) Total number of cells in the human SCN and SDN. The SCN in homosexual men contains 2.1 times as many cells as the SCN in the reference group of male subjects and 2.4 times as many cells as the SCN in heterosexual AIDS patients. (**c**) The number of vasopressin neurons in the human SCN (the SDN does not contain vasopressin-producing cells). The SCN in homosexual men contains, on average, 1.9 times as many vasopressin neurons as the SCN in heterosexual AIDS patients. Note that the SCN of heterosexual individuals who died of AIDS contains fewer vasopressin cells than the SCN of the subjects from the reference group [From Swaab and Hofman (1990); Fig. 2, with permission]

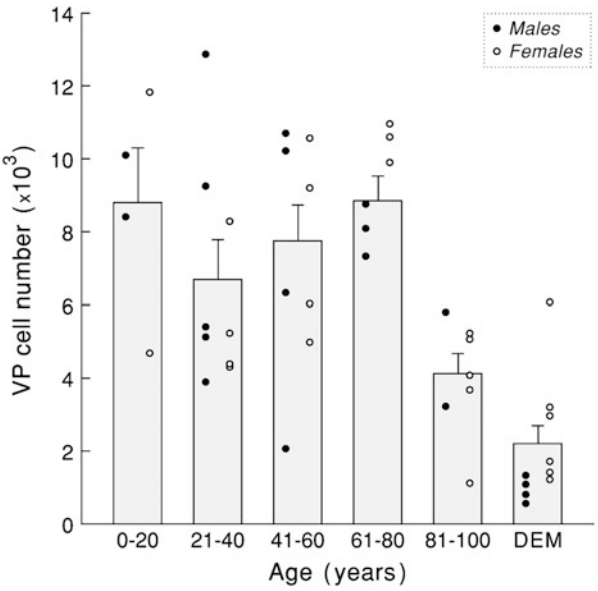


Fig. 4.9 Number of vasopressin (AVP)-expressing neurons in the SCN. Note the low values in the 81- to 100-year-old group and the very low numbers in the AD patients (DEM) that were 78 ± 5 years of age. The decreased number of cells expressing VP is considered to be an indication of low metabolic activity of the SCN in old people and AD patients and the changes in the SCN in AD are held responsible for sleep disturbances and nightly restlessness [Based upon Swaab et al. (1987) Fig. 1, with permission]. The variability is largely due to circadian and circannual changes (see Figs. 4.5, 4.6, and 4.7)

periods have already been observed in subjects aged 50–60 (Wu and Swaab 2007), whereas a reduction in SCN volume and number of AVP-expressing neurons is only present from the age of 80 years onward (Fig. 4.9) (Swaab et al. 1985, 1993; Hofman 1997). The observed AVP-expressing neurons loss in the SCN at later ages may, therefore, only be a relatively late correlate of functional changes in the biological clock that appear much earlier.

The pineal hormone melatonin is involved in the regulation of circadian rhythms and feeds back to the central biological clock, the hypothalamic SCN, via MTs. Supplementary melatonin is considered to be a potential treatment for age-related circadian disorders. Alterations of the MT1 receptor in the SCN during aging were investigated by immunocytochemistry in postmortem human brain material; the number and density of AVP- and VIP-expressing neurons in the SCN did not change, but the number and density of MT1-expressing neurons in the SCN were decreased in aged versus young controls (Fig. 4.10) (Wu et al. 2007).

It has been demonstrated in aged rats (up to 37 months old) that both overt sleep–wake rhythms (Witting et al. 1993) and AVP-expressing neurons in the SCN (Lucassen et al. 1995) can be restored by enhancing the amount of environmental light. A similar degree of plasticity was also observed in the human circadian

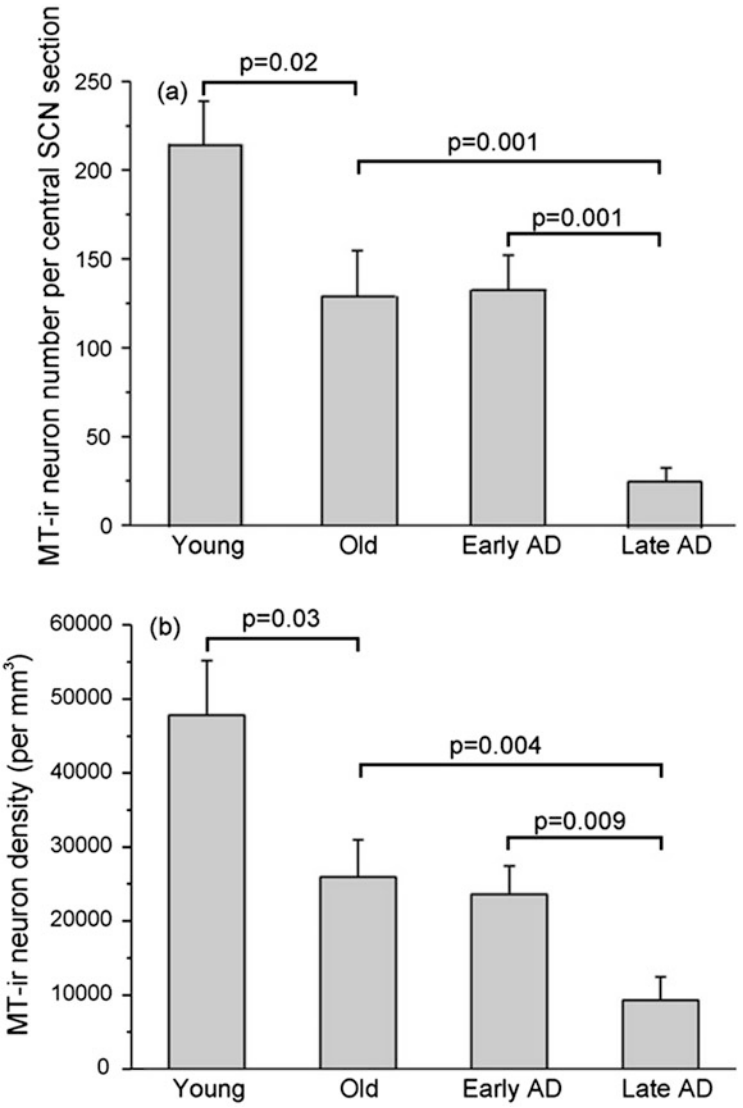


Fig. 4.10 (**a**) MT1-immunoreactive (MT1-ir) neuron number per central SCN section and (**b**) MT1-ir neuron density in the central SCN section in young controls, aged controls (Braak stage 0), subjects in early AD stages (Braak stages I–II; preclinical AD), and late stage AD patients (Braak stages V–VI). Note that both MT1-ir neuron density and number are decreased in the old controls and preclinical "AD" subjects and are more dramatically decreased in the late stage clinical AD patients [From Wu et al. (2006b); Fig. 2, with permission]

timing system. Improvement of the sleep–wake rhythm of older people and patients with dementia was demonstrated by application of a variety of potent modulators of the circadian timing system such as bright light, melatonin, and physical activity (Van Someren et al. 2002) (see also below).

4.2 Input SCN

The RHT is the principal pathway mediating the entraining effects of light on the circadian pacemaker, the SCN. Indeed, suprasellar tumors with compression of the optic chiasm leading to permanent visual field defects are associated with reduced AVP but not with reduced VIP immunoreactivity in the SCN, which raises the possibility that selective impairment of the SCN contributes to the sleep–wake disturbances these patients experience (Borgers et al. 2011, 2013).

The RHT is made up of retinal ganglion cells, which contain pituitary adenylate cyclase-activating polypeptide (PACAP) and co-store glutamate. Light activates these cells, which directly innervate the SCN via the photopigment melanopsin in the PACAP cells. PACAP interacts with glutamate signaling during the light-induced phase shift. At the same time, the retina itself possesses intrinsic circadian oscillations, exemplified by diurnal fluctuations in visual sensitivity, neurotransmitter levels, and outer segment turnover rates (Hannibal 2006). The human RHT was studied by means of postmortem tracing. Remarkably, up to 6–8 h after the death of the patients, the individual neurons are still capable of actively taking up tracer molecules and transporting them over relatively large distances. The RHT appeared to leave the optic chiasm and enter the hypothalamus both medially and laterally of the SCN. The density of the RHT fibers decreases from rostral to caudal (Fig. 4.11) (Dai et al. 1998b). The RHT terminates predominantly in a zone of the SCN that contains VIP neurons (Moore 1992) but does not only contact VIP but also neurotensin cells in the SCN. In addition, some AVP-expressing neurons are innervated by the RHT in the ventral part of the SCN. Only few projections to the dorsal part of the SCN and the ventral part of the anterior hypothalamus were found (Dai et al. 1998b). Lateral RHT projections reach the ventral part of the ventromedial SON. These fibers may take part in diurnal fluctuations of AVP release. Lateral RHT projections also innervate the area lateral to the SCN. No projections to other hypothalamic areas were observed (Dai et al. 1998b). These data generally confirmed Sadun et al.'s observations with paraphenylenediamine, which stains remnants of degenerated axons in patients with a lesion of the optic nerve (Sadun et al. 1983), and confirmed Friedman et al.'s observations following DiI staining of the RHT in intact human brains (Friedman et al. 1991). Dai et al. (1998b) could, however, not confirm the innervation of the hypothalamic PVN by human RHT that was described by the degeneration technique (Schaechter and Sadun 1985). This technique may, however, have been confounded by lesions that were not restricted to the optic nerve.

Compared to monkeys and other animals, the human SCN has a very large population of neurotensin cells and a large population of neuropeptide Y neurons, which obscure a geniculo-hypothalamic tract that contains the same peptide, provided this tract is present in all humans (Moore 1992). In addition, serotonin innervates the SCN, and histamine, the neurotransmitter produced in the tuberomamillary nucleus, is necessary for the circadian rhythmicity of adrenocorticotropic hormone (ACTH) release, food intake, drinking, and the sleep–

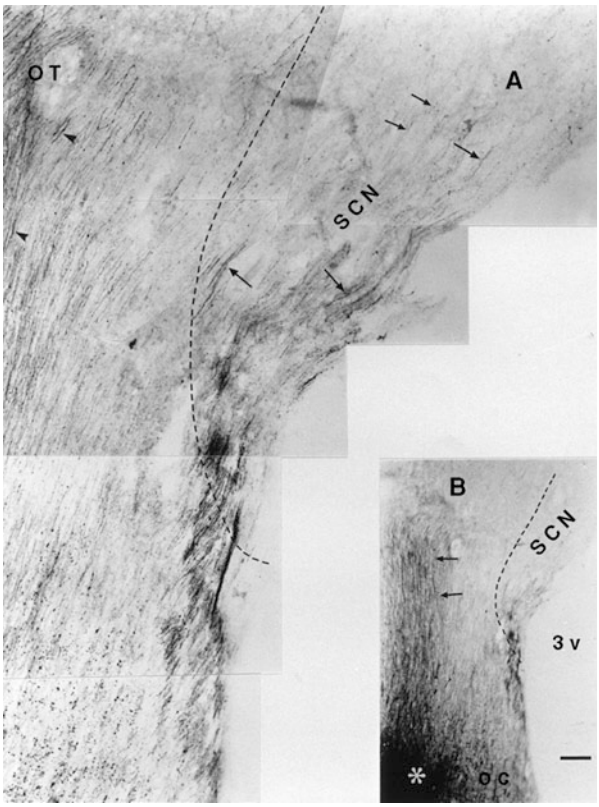


Fig. 4.11 Postmortem tracing of the retinohypothalamic tract. Anterior level of the suprachiasmatic nucleus (SCN) showing the injection spot (*asterisk*, **b**) in the optic nerve. Many labeled fibers (**a**) can be seen to course along the wall of the third ventricle (3V) and project to the SCN (*arrows*). Many fibers (**b**) also extend to the optic tract (*arrows*). (**a**) shows the high magnification of the area in (**b**) (*arrowheads*) and shows more clearly labeled fibers in the optic nerve and ventral part of the SCN. The morphology of labeled fibers is clearly visible. *Dashed lines* in (**a**) and (**b**) represent the lateral border of the SCN. *Scale bar* = 40 mm for (**a**), 150 mm for (**b**) [From Dai et al. (1998a, b, c); Fig. 5, with permission]

wakefulness cycle. In fact, histamine can phase-shift circadian rhythms and some authors even consider it to be the final neurotransmitter in the entrainment of the SCN (Eaton et al. 1995; Hannibal 2002).

Another important input to the SCN which influences circadian and circannual functions is melatonin, produced by the pineal gland and acting on the SCN through MT receptors (Wu et al. 2006b, 2013).

Finally, it should be noted that the rest–activity cycle and meals influence rhythmic endocrine changes as well (Swaab 2003). Circadian rhythms in the endocrine system are covered in detail in Chap. 2.

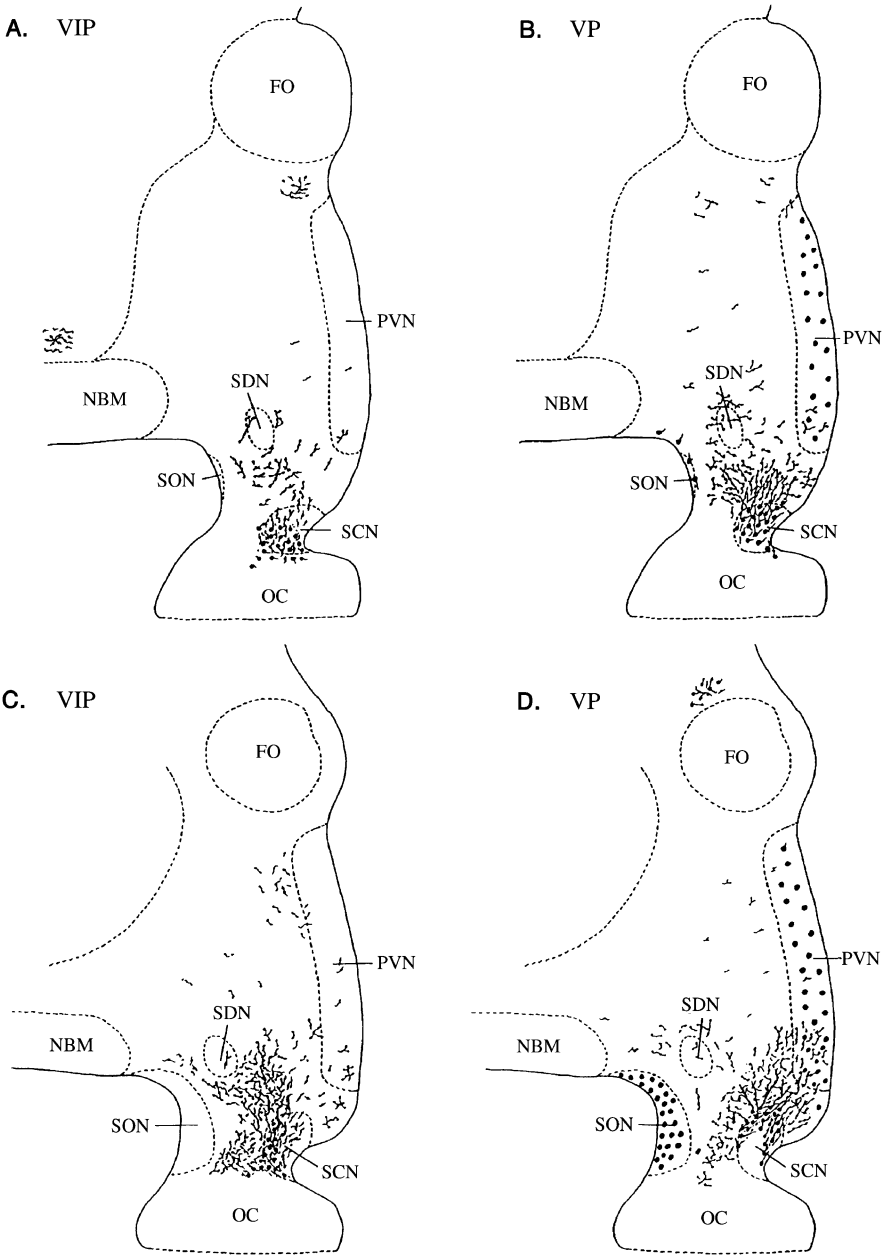
4.3 Output SCN

Many, if not all, brain areas are directly or indirectly functionally affected by the diurnal fluctuations of the nervous output of the SCN and by melatonin fluctuations from the pineal gland. Immunocytochemical observations show that AVP and VIP fibers innervate the SCN itself as well as a number of other hypothalamic areas,

including the contralateral SCN, and these observations match very well with postmortem tracing following an injection in the human SCN (Dai et al. 1998a). The densest projections from the human SCN first reach the area between the SCN and the anteroventral part of the hypothalamic PVN, the anteroventral hypothalamic area (Dai et al. 1998a). Immunocytochemical staining showed AVP and VIP fibers in these areas, some of which run anteriorly and enter the anteroventral part of the periventricular nucleus and of the PVN (Fig. 4.12) (Dai et al. 1997). Many SCN fibers continue in the posterior direction and innervate the zone below the PVN, or they reach the ventral PVN (Dai et al. 1998a). The dense network of AVP- and VIP-positive fibers in the sub-PVN zone (Dai et al. 1997) is in agreement with this observation. The SCN fibers in the ventral PVN innervate AVP and corticotropin-releasing hormone (CRH) neurons. This mainly concerns AVP fibers (Dai et al. 1997). The SCN fibers innervating the PVN may directly or indirectly (see below) provide an anatomical basis for the strong influence of the SCN on hormone secretion. Another extensive projection courses posteriorly and passes close to the third ventricle to reach the dorsomedial nucleus (DMN) of the hypothalamus. Most fibers innervating the DMN are concentrated in its ventral part (Dai et al. 1998a) and VIP fibers were more abundant than AVP fibers (Dai et al. 1997). Also in the human brain, the DMN projects to the PVN (Dai et al. 1998c). The SCN thus also influences PVN functions in an indirect way, via the DMN. Injections in the dorsal part of the SCN showed more extensive projections to the PVN than those placed in the ventral part of the SCN. Tracing or immunocytochemistry showed the presence of only a few fibers in the ventromedial nucleus (VMN) (Dai et al. 1997, 1998a).

In rodents there is a strong projection from the SCN to the SON with both inhibitory (GABAergic) and excitatory (glutaminergic) components that may also be responsible for the circadian rhythmicity in the SON. Such connections have, however, not been shown in the human brain, although SCN fibers come very close to the SON (Dai et al. 1997) and possibly even contact SON dendrites or interneurons. In addition, the lateral RTH tract projections that innervate the ventral part of the SON (Dai et al. 1998b) may impose a diurnal rhythm on AVP release. Transcriptome-wide analysis of the human brain demonstrated a rhythmic rise and fall of gene expression in regions outside of the SCN, including the dorsolateral prefrontal cortex, the anterior cingulate cortex, the hippocampus, the amygdala, the nucleus accumbens, and the cerebellum. Some 700 transcripts in each region showed 24-h cyclic patterns in controls, and more than 100 transcripts exhibited consistent rhythmicity and phase synchrony across regions. Among the top-ranked "rhythmic" genes were the canonical clock genes BMAL1 (ARNTL), PER1-2-3, NR1D1 (REV-ERBa), DBP, BHLHE40 (DEC1), and BHLHE41 (DEC2) (Li et al. 2013).

The diurnal fluctuations in central functions are not only caused by the neuronal output of the SCN, but also by the action of melatonin on the brain. The pineal gland is innervated by sympathetic noradrenergic fibers. This polysynaptic pathway starts in the SCN and passes the cervical spinal cord. This explains the prevalence of decreased sleep quality in individuals with tetraplegia that is due to a cervical spinal cord lesion between C4 and C7 and the complete absence of the evening



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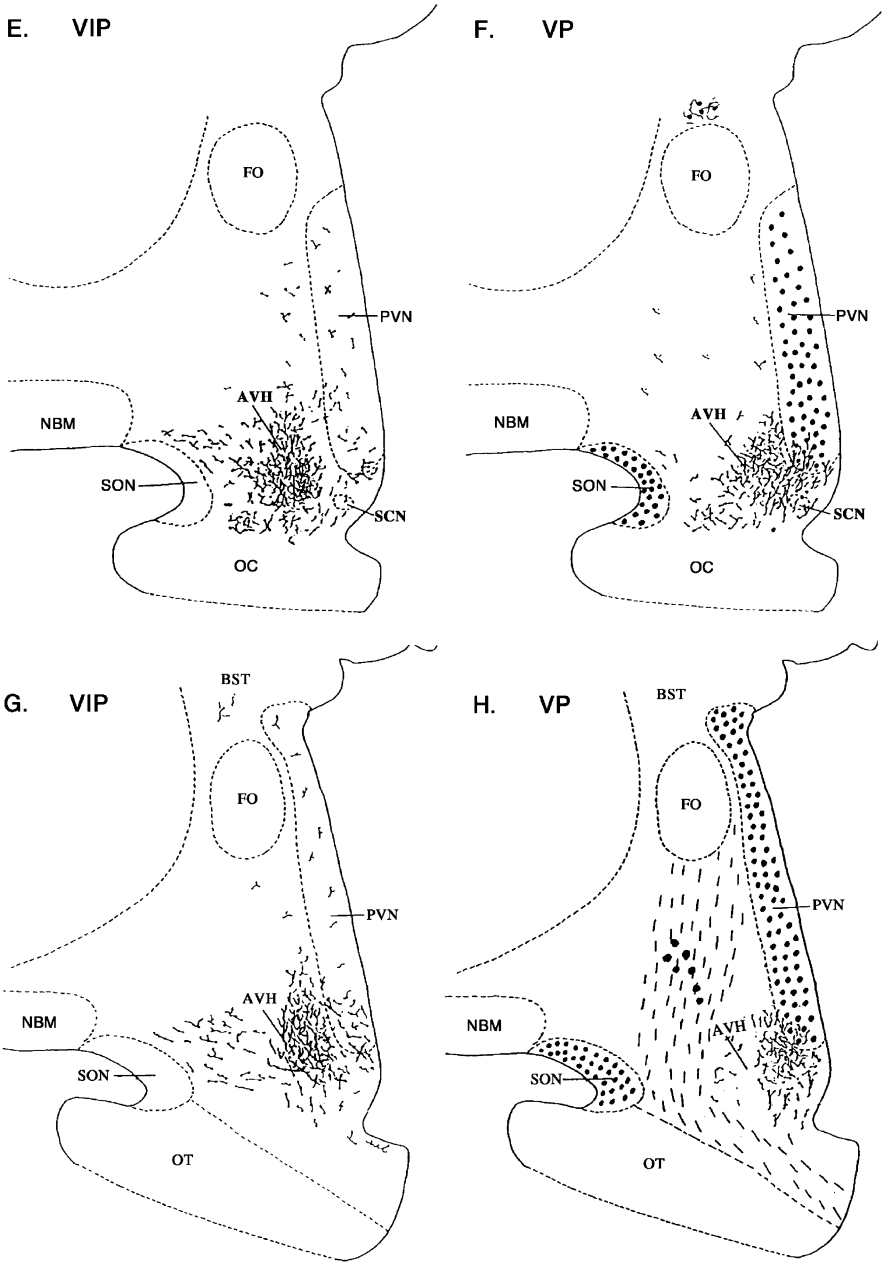

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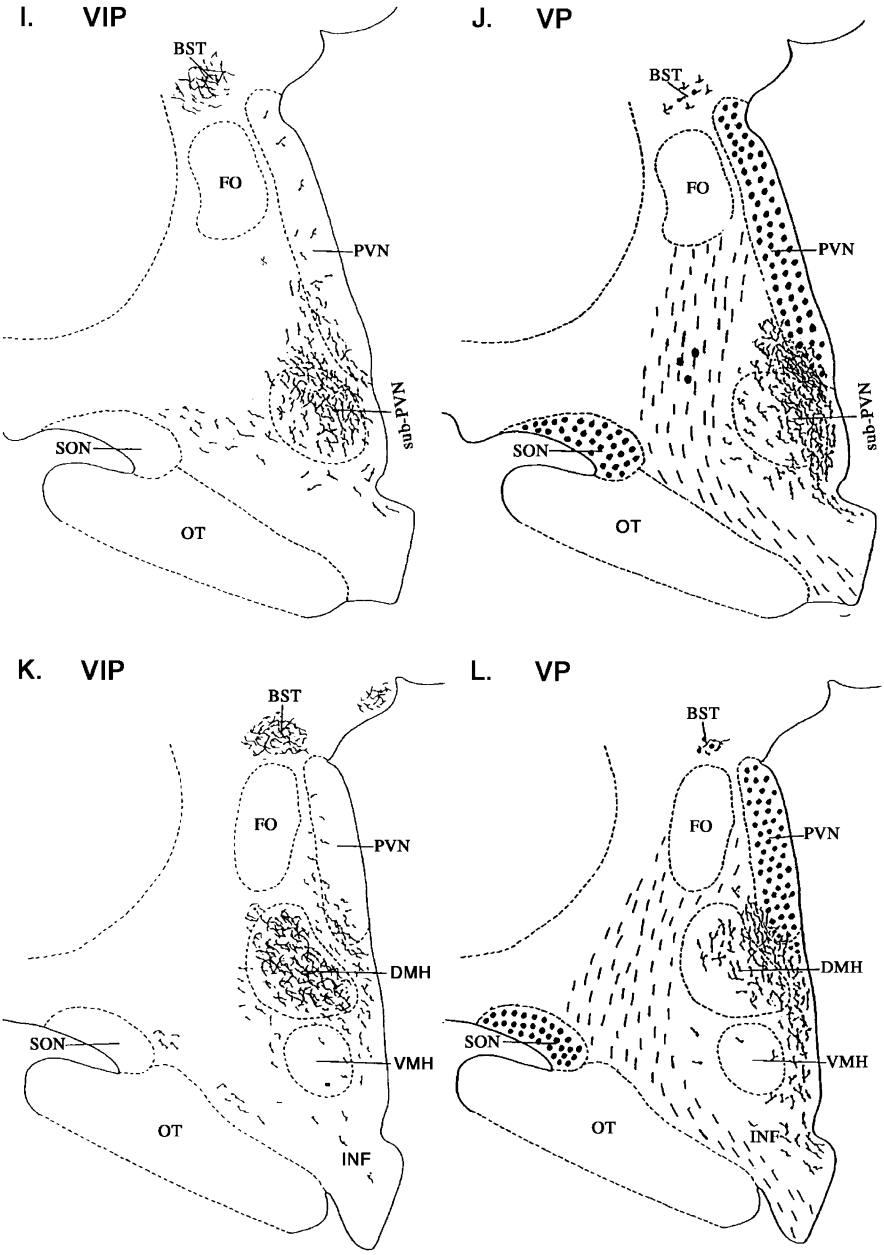

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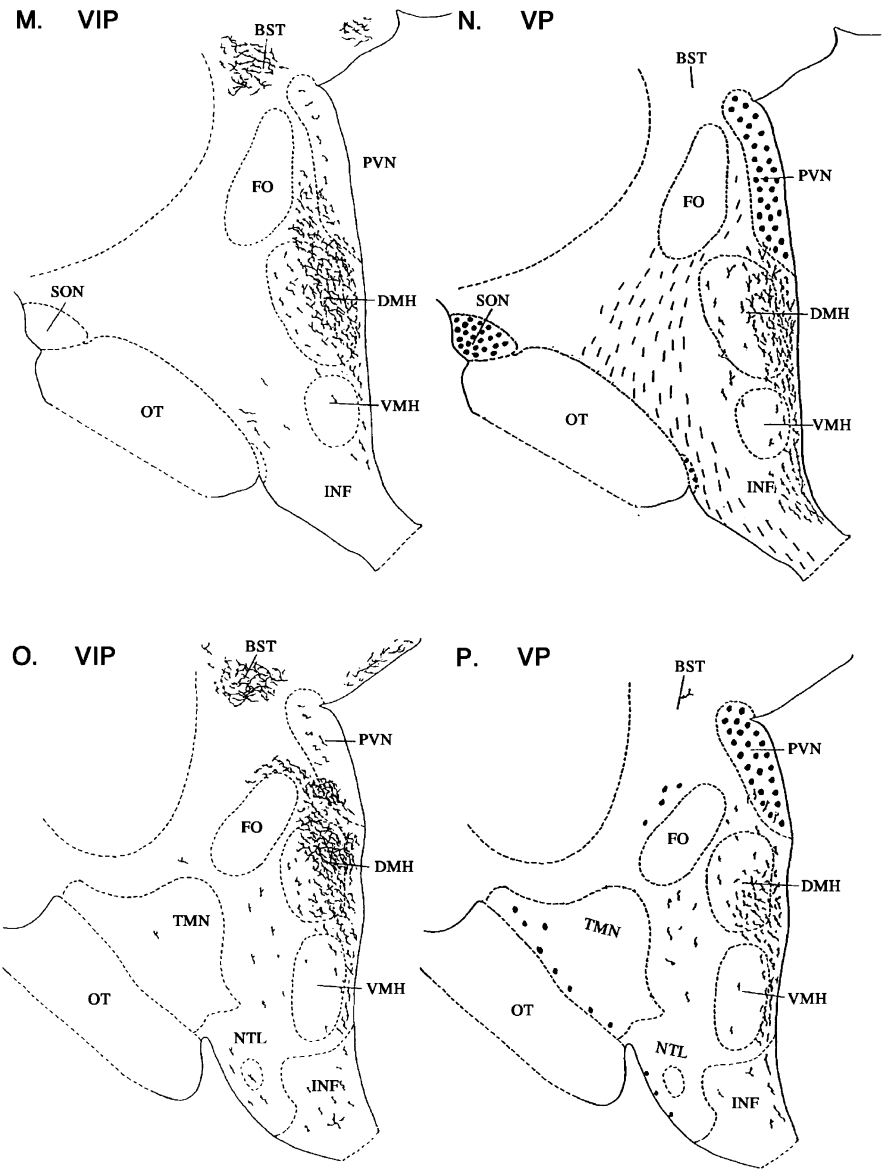

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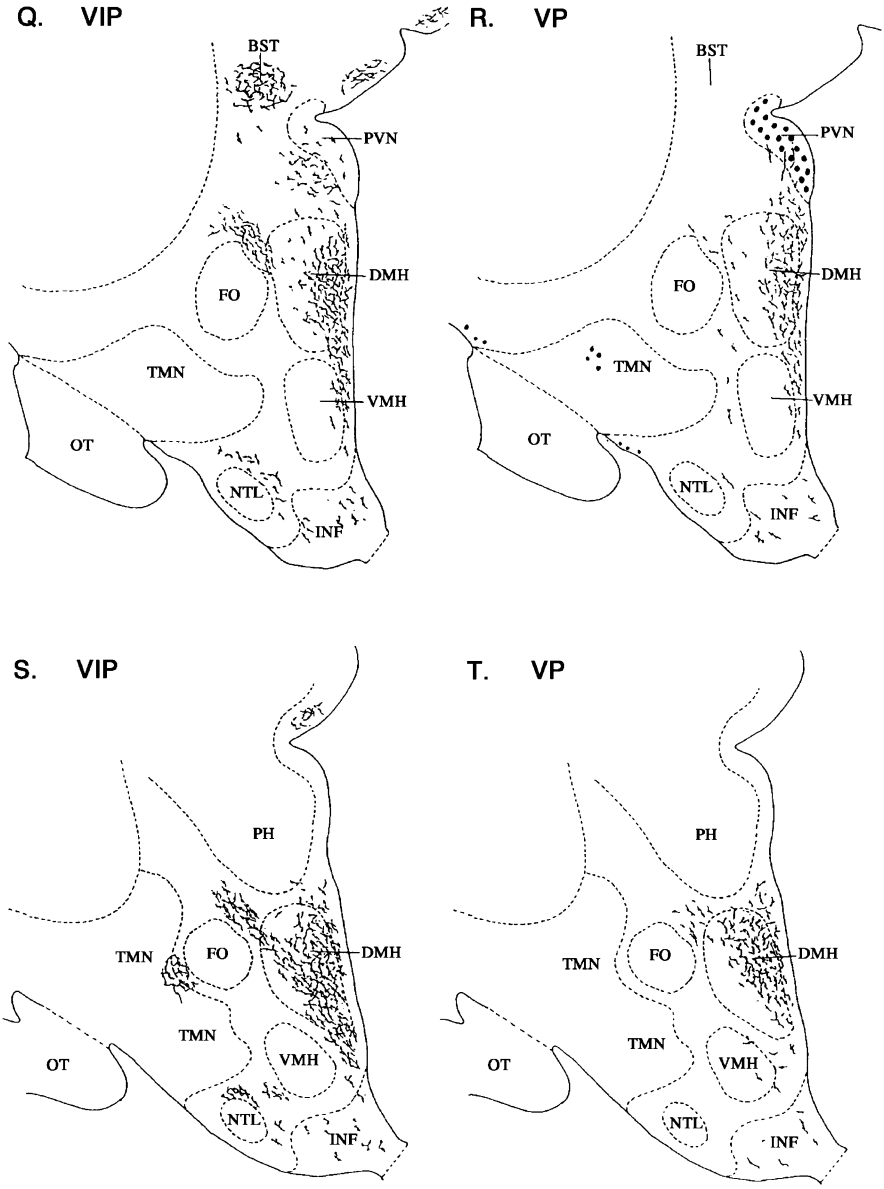

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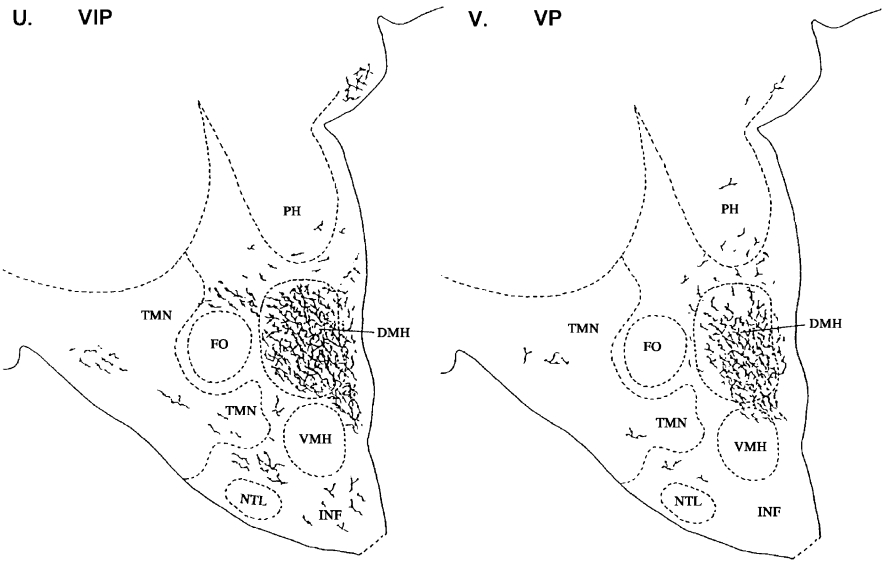

Fig. 4.12 A series of line drawings arranged from rostral to caudal (**a**–**v**) to illustrate schematically the location of vasoactive intestinal polypeptide (VIP) and vasopressin (VP) cell bodies and fibers in a human hypothalamus (case no. 96-010). The *dots* correspond to the position and density of the cell bodies. *Short lines* (in **j**, **l**, **n**) illustrate the area through which the fibers of VP magnocellular cell bodies pass. *AVH* anteroventral hypothalamic area, *BST* bed nucleus of the stria terminalis, *DMH* dorsomedial nucleus of the hypothalamus, *FO* fornix, *INF* infundibular nucleus, *MB* mamillary body, *NBM* nucleus basalis of Meynert, *NTL* lateral tuberal nucleus, *OC* optic chiasm, *POA* preoptic area, *PVN* paraventricular nucleus, *PH* posterior hypothalamic nucleus, *PEN* periventricular nucleus, *SCN* suprachiasmatic nucleus, *SDN* sexually dimorphic nucleus of preoptic area (=INAH-1), *SON* supraoptic nucleus, *sub-PVN* area below paraventricular nucleus, *TMN* tuberomamillary nucleus, *VMN* ventromedial nucleus, *VP* vasopressin, *VIP* vasoactive intestinal polypeptide [From Dai et al. (1997); Fig. 2, with permission]

increase in melatonin concentration in this group of patients (Verheggen et al. 2012).

4.4 SCN in Neuropsychiatric Diseases

4.4.1 Alzheimer's Disease

The disruption of circadian rhythms and the increased incidence of disturbed sleep in humans during aging (Van Someren et al. 2002) are accompanied by age-related alterations in the neural organization of the SCN, a decreased photic input to the clock, and a dramatic decrease in peptide synthesis in the SCN of Alzheimer's Disease (AD) (Liu et al. 2000; Swaab 2004b). In addition, the presence of

pretangles (Swaab et al. 1992; van de Nes et al. 1998) and tangles (Stopa et al. 1999) in the SCN of AD patients shows that this structure is affected by the disease process. However, diffuse amyloid plaques are only seldom found in this nucleus (van de Nes et al. 1998; Stopa et al. 1999). Stopa et al. (1999), for example, reported that the SCN of AD patients, who tend to suffer from sleep disruption and other circadian rhythm disturbances, is severely damaged and exhibits a dramatic loss of AVP- and neurotensin-expressing neurons and a corresponding increase in the glial fibrillary acidic protein (GFAP)-stained astrocytes. Harper et al. (2008) have observed, in postmortem material, in relation to antemortem clinical data, that specific loss of SCN neurotensin neurons was associated with loss of activity and temperature amplitude without increase in activity fragmentation. In addition, the loss of SCN AVP-expressing neurons was associated with increased activity fragmentation but not with loss of amplitude. Harper et al.'s data provide information on the role of neuronal subpopulations in subserving the SCN function and the utility of AD.

The immunocytochemical data showing decreased activity of the SCN in AD (Figs. 4.9 and 4.13) have been confirmed by in situ hybridization. The total amount of AVP-mRNA in the SCN of AD patients was three times lower than in age- and gender-matched controls. In addition, the AVP-mRNA-expressing neurons in the SCN showed a marked day–night difference in controls under the age of 80 years. The amount of AVP-mRNA was more than three times higher during the day than at night, whereas no clear diurnal rhythm of AVP-mRNA was observed in AD

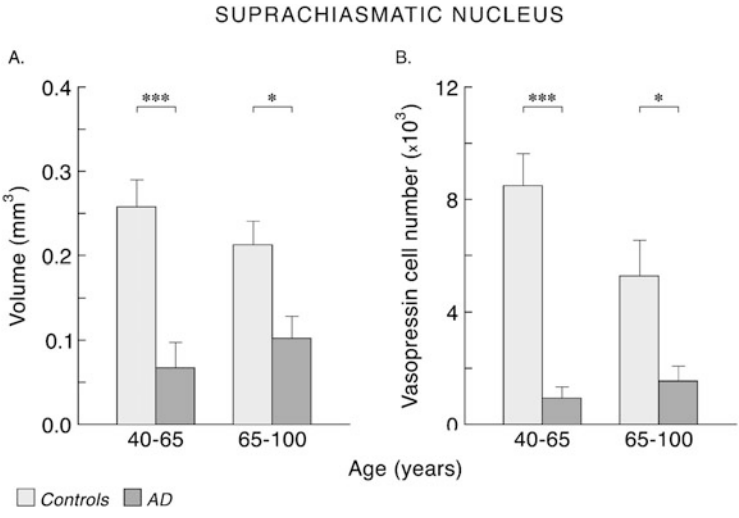

Fig. 4.13 In both presenile ($n = 7$) and senile ($n = 8$) Alzheimer patients the volume of the vasopressin subnucleus of the SCN (**a**) and the number of vasopressin-expressing neurons (**b**) are significantly decreased when compared to young ($n = 14$) or old ($n = 9$) age-matched controls. In presenile Alzheimer patients only 10 % of the number of neurons expressing vasopressin in controls is found. ***$p > 0.001$; *$p > 0.02$ (Mann–Whitney-*U*-test) [From Swaab (2004a); Fig. 29.7, with permission]

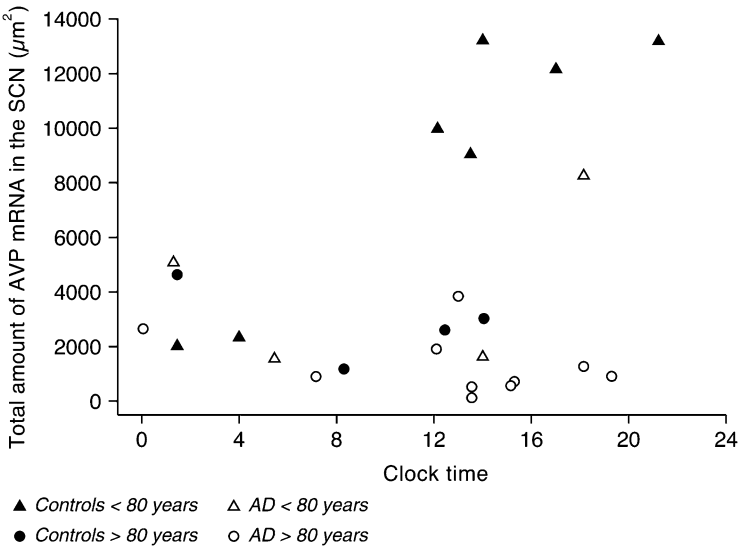

Fig. 4.14 Day–night fluctuations in vasopressin (AVP)-mRNA in the suprachiasmatic nucleus (SCN) expressed as masked area of silver grains in controls and in Alzheimer patients (AD). Note that at any moment of the day the values for AD patients are lower than those for controls [From Liu et al. (2000); Fig. 3, with permission]

patients (Fig. 4.14) (Liu et al. 2000). Only in female presenile Alzheimer patients was a significant decrease in the number of VIP-expressing neurons in the SCN found (Zhou et al. 1995). The AVP and VIP data support the idea that damage to the SCN is the underlying anatomical substrate for the clinically often observed disturbances in circadian rhythmicity in AD.

It was found that the number and density of AVP- or VIP-expressing neurons in the SCN did not change in aged control subjects compared to young control subjects, but the number and density of MT1-expressing neurons in the SCN were decreased. In addition, both MT1-expressing neurons and AVP- or VIP-expressing neurons were strongly diminished in the final neuropathological stages of AD (Braak stages V–VI), but not in the earliest stages (Braak stages I–II), compared to aged control subjects (Braak stage 0) (see Fig. 4.10). These data suggest that the MT1-mediated effects of melatonin on the SCN are disturbed during aging and even more so in late stage AD, which may contribute to the clinical circadian disorders and to the efficacy of therapeutic melatonin administration under these conditions (Wu et al. 2007).

Day–night fluctuations in pineal melatonin production are already disrupted in the preclinical AD stages (Braak stages II–III) (Fig. 4.15) (Wu et al. 2003), which was proposed to be due to a disruption of the SCN functions. The diurnal fluctuation in the pineal gland in the expression of the clock genes *hBmal1*, *hCry1*, *hPer1* and in the expression of the β-adrenergic receptor is lost in the pineal gland, and AVP-mRNA levels decrease in the SCN of both preclinical (Braak stages I–II)

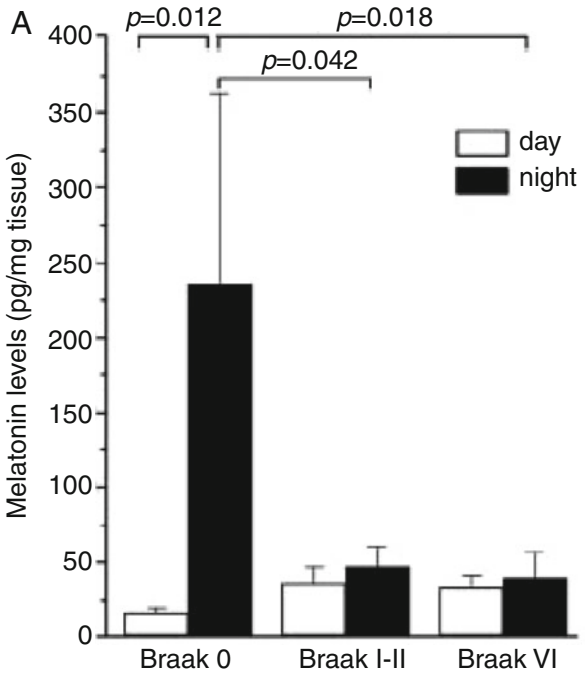

Fig. 4.15 The day–night difference in pineal melatonin content is present in Braak stage 0 controls, but disappears in Braak stage I–II and is also absent in Braak stage VI [From Wu et al. (2003); Fig. 3, with permission]

and clinical (Braak stages V–VI) AD subjects, indicating that the activity of the SCN and of the pineal gland is reduced very early on in AD pathogenesis (Wu et al. 2006a).

In a long-term, double-blind, placebo-controlled, randomized trial performed with 189 residents in the Netherlands, 87 % of whom had dementia, a combined treatment of light and melatonin was found to increase sleep efficiency and to improve nocturnal restlessness (Riemersma-van der Lek et al. 2008). Contrary to treatment with hypnotics, the improvement of sleep following these treatments is without adverse effects and even results in the improvement of mood, performance, daytime energy, and quality of life. All these data support the hypothesis that increased stimulation of the brain can improve or even restore the decreased neuronal activity (Swaab et al. 2002).

Concluding, there is a loss of function in both the SCN and the pineal gland in aging and in the earliest presymptomatic stages of AD, while these alterations in the circadian system become more pronounced in the later stages of AD. The SCN can functionally be stimulated in AD by enhanced environmental light,

4.4.2 Other Neurodegenerative Disorders

Huntington's disease (HD) has a number of features that suggest dysfunction of the SCN, such as a disturbed circadian secretion patterns of cortisol and melatonin, the frequent occurrence of sleeping disorders and the presence of huntingtin-positive cytoplasmic inclusion bodies in the SCN of some patients (Aziz et al. 2008). Studies in HD patients yielded the information that the SCN contained 85 % less VIP-expressing neurons and 33 % less AVP-expressing neurons. However, the total amount of VIP- or AVP-mRNA was unchanged. No significant changes were observed in the number of MT-1- or MT-2 receptor-expressing neurons. These findings suggest that the disorders in sleep and other circadian rhythms in HD patients may at least partly arise from SCN dysfunction (van Wamelen et al. 2013a) and that there are post-transcriptional neuropeptide changes in the SCN of HD patients. However, the expression of the prohormone convertases PC1/3 and PC2 expressions were not different between HD and control cases (van Wamelen et al. 2013b).

In the SCN of one of two patients with *hippocampal sclerosis*, Stopa et al. (1999) found an increased ratio of astrocyte to neuron. In both these patients the density of AVP- and neurotensin-expressing neurons seemed to be below average, although more subjects are required to be able to draw a proper conclusion. In addition, there are indications that the seizures seen in hippocampal sclerosis affect circadian rhythms (Quigg et al. 1999).

Ozawa et al. (1993) described a patient with *Shy–Drager syndrome (multisystem atrophy)*, who exhibited nocturnal polyuria associated with decreased urinary-specific gravity and a reversed circadian rhythm of AVP, suggesting that the SCN was affected. Subsequently, postmortem evidence indeed provided a disorder in the SCN of this disease. The patient had a decreased number of AVP-expressing neurons, together with gliosis, in the SCN. In addition, the AVP-expressing neurons in the SCN of this patient were smaller than those of the control subjects. Moreover, there was an observation that patients with multisystem atrophy have decreased early morning cortisol levels, which indicates a functional alteration of the SCN (Ozawa et al. 2001). Furthermore, a decrease in the nightly plasma AVP levels has been confirmed in a sample of 13 patients with multisystem atrophy (Ozawa et al. 1998), and the physiological nocturnal fall of body core temperature is blunted in multisystem atrophy patients. The lack of a decrease in body temperature in these patients—possibly caused by a defect in the SCN—distinguishes them from Parkinson patients (Pierangeli et al. 2001).

In the SCN of three *Pick Disease patients*, Stopa et al. (1999) have found a decreased density of AVP- and neurotensin-expressing neurons, which are changes similar to those observed in AD. In a 1-year-old boy with X-linked lissencephaly with abnormal genitalia, who had absent circadian variation in rhythms of sleep and core temperature, the SCN was not identified, in spite of the use of anti-AVP (Miyata et al. 2009).

4.5 Depression

The human SCN not only shows circadian but also circannual variations in neuronal activity (see above, Fig. 4.6), which is supposed to be related to circadian and circannual fluctuations in mood and to sleeping disturbances in depression (van Londen et al. 2001). The symptoms of depression fluctuate over the day, and the stress response, too, is strongly influenced by the time of the day. The activity of the SCN is directly influenced by light and light therapy is found to be affected in depression. Seasonal affective disorder (SAD) is more prevalent in the northern states of the USA than in the southern states (Miller 2005). So there is a close relationship between light, the SCN, and mood. Since polymorphisms in the clock genes and in lithium target GCK3 are associated with dysfunctional circadian rhythm and susceptibility to mood disorders, in particular in SAD and bipolar disorder (BD) (Lamont et al. 2007), the SCN may also play a causal role in depression at least in subgroups of patients.

Zhou et al. found a disorder of SCN function that is characterized by an increased number of AVP-expressing neurons, a decreased amount of AVP-mRNA in this nucleus, and diminished circadian fluctuation of AVP-mRNA in the postmortem brain of depressed patients (Fig. 4.16) (Zhou et al. 2001). Decreased activity of the SCN in depression is presumed to be, at least partly, due to the increased circulating plasma cortisol levels observed in depression

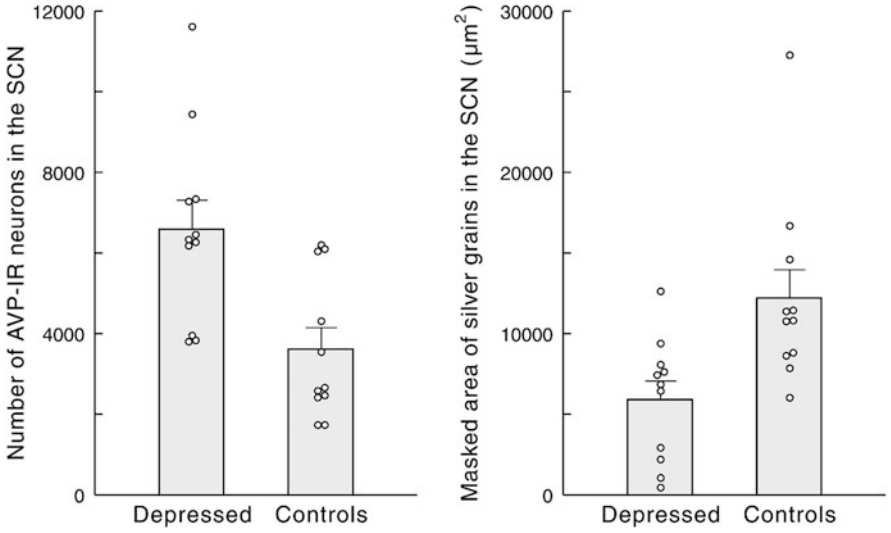

Fig. 4.16 The number of arginine vasopressin-immunoreactive (AVP-IR) neurons (**a**) and the mask area of silver grains of the AVP-messenger RNA (**b**) in the suprachiasmatic nucleus (SCN) in control subjects ($n = 11$) and depressed subjects ($n = 11$). The *error bars* indicate the SD. Note the change in the balance between the presence of more AVP and less AVP-messenger RNA in depression. There is probably a disorder of the transport of AVP that leads to accumulation of the peptide, in spite of the decreased production rate [From Zhou et al. (2001); Fig. 2, with permission]

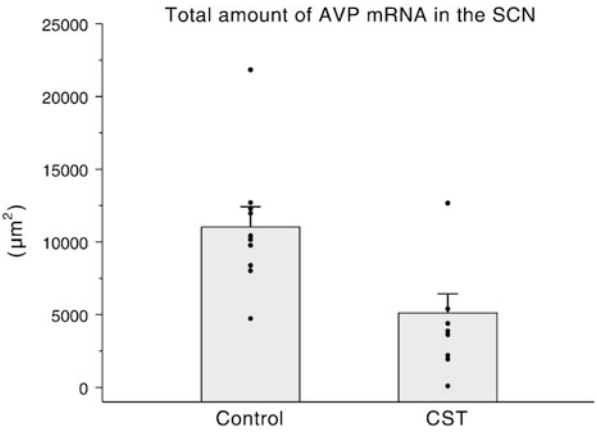

Fig. 4.17 Estimated total amount of AVP-mRNA in the suprachiasmatic nucleus (SCN) (expressed as masked area of silver grains) of the controls and the corticosteroid-exposed subjects (CST). The *bars* and *error lines* represent the mean and standard error of the mean (SEM) [From Liu et al. (2006); Fig. 2, with permission]

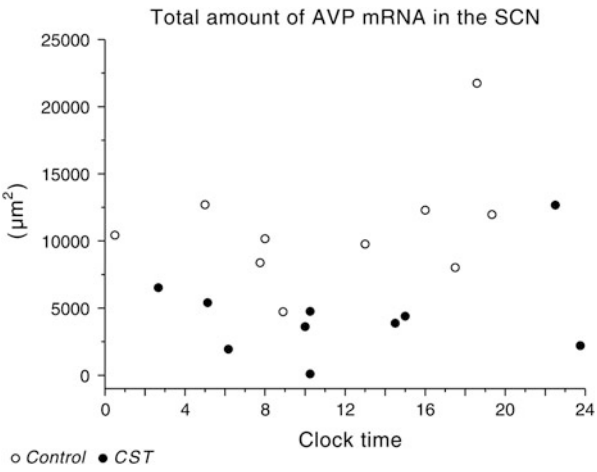

Fig. 4.18 Day–night fluctuation in the total amount of AVP-mRNA of the SCN in controls and in the glucocorticoid-exposed subjects (CST). Note that at any moment of the day the values for CST are lower than those of controls [From Liu et al. (2006); Fig. 3, with permission]

patients, since corticosteroids inhibit the mRNA expression of AVP in the SCN (Figs. 4.17 and 4.18) (Liu et al. 2006). In a transcriptome-wide analysis of the human brain it was also found that the day–night fluctuations were much weaker in the brains of major depression disorder patients due to shifted peak timing and potentially disrupted phase relationships between individual circadian genes (Li et al. 2013).

The number of MT1-immunoreactive (ir) cells and AVP- and/or VIP-ir cells were found to be increased in the central SCN in depression, while the number of MT2-ir cells was not altered (Wu et al. 2013). In addition, the number of MT1-ir cells, but not MT2-ir cells, was negatively correlated with age at onset of depression, while positively correlated with disease duration. MT1 receptors appeared to be specifically increased in the SCN of depressed patients and may increase during the course of the disease. These changes may be involved in circadian disorders and may contribute to the efficacy of MT receptor agonists or melatonin in depression.

Moreover, these data suggest that MT receptor agonists for depression should be selectively targeted toward the MT1 receptor (Wu et al. 2013).

A major question is how light therapy may work in depression. Animal data have shown that AVP neurons of the SCN exert an inhibitory influence on CRH neurons in the PVN (Kalsbeek et al. 1992). Depressed patients have a deficient SCN (Zhou et al. 2001), which may subsequently fail to inhibit sufficiently the CRH neurons in the PVN of depressed patients. Such an impaired negative feedback mechanism may lead to a further increase in the activity of the HPA axis in depression. Both the resulting higher CRH and cortisol levels may contribute to the symptoms of depression. Light therapy may activate the SCN, directly inducing an increased synthesis and release of AVP that will inhibit the CRH neurons. However, it should be noted that human beings are diurnal creatures, whose mode of interaction between SCN-AVP neurons and the PVN-CRH neurons might be different from that of the nocturnal rat, especially because of the fact that opposite actions of hypothalamic AVP have recently been observed on the circadian corticosterone rhythm in nocturnal versus diurnal species (Kalsbeek et al. 2008). The exact mechanism of the action of light in depressed patients thus deserves further study.

4.6 Hypertension

In a postmortem study of the SCN of primary hypertension patients who had died due to myocardial infarction or brain hemorrhage, and in comparison with controls who had normal blood pressure, it was found that the immunoreactivity staining for the three major neuronal populations of the SCN, i.e., AVP, VIP, and neurotensin, was reduced by more than 50 % in hypertension compared with controls (Fig. 4.19) (Goncharuk et al. 2001).

In hypertensive patients an extremely high expression of CRH was also observed in all parts of the hypothalamic PVN (Fig. 4.19). In addition, in contrast to the controls, the hypertensive patients had a very high number of CRH fibers running from the most rostral part of the PVN to the median eminence and innervating the caudal part of the SCN. A quantitative evaluation showed that the area covered by CRH fibers in the SCN of hypertensive patients was about three times larger than that in the SCN of controls. Moreover, a clear negative correlation was found between the area of CRH fibers and the number of AVP- or neurotensin-expressing neurons within the SCN (Goncharuk et al. 2007). These data indicate a serious dysregulation of the SCN in hypertensive patients. Such a disturbance may cause a harmful hemodynamic imbalance with a negative effect on circulation, especially in the morning, which is when the inactivity–activity balance changes. The difficulty in adjusting from inactivity to activity might be involved in the morning clustering of cardiovascular events. Interestingly, a transgenic hypertensive mouse strain (TGR(mRen2)27) showed an altered light-entrainment response, accompanied by suppressed c-fos-mRNA expression in the SCN (Lemmer et al. 2000), indicating a possible primary involvement of the SCN in hypertension.

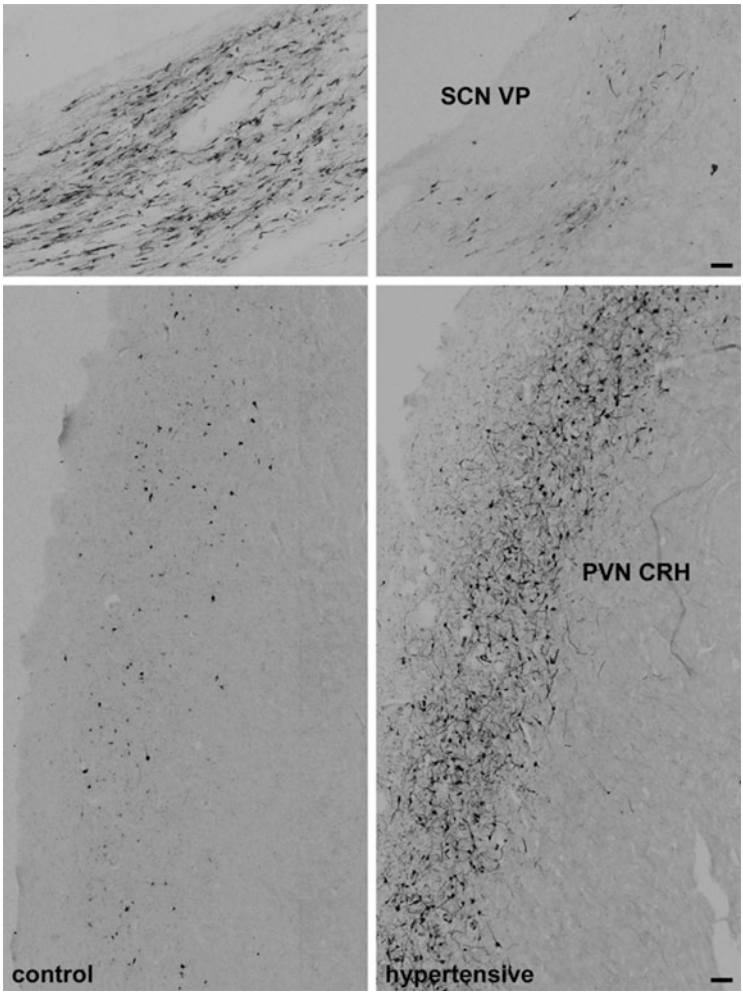

Fig. 4.19 Coronal sections of the human hypothalamus stained for vasopressin (VP) (*upper images*) [area of the suprachiasmatic nuclei (SCN)], or for corticotropin-releasing hormone (CRH) (*lower images*) [area of the paraventricular nuclei (PVN)]. Sections on the *left side* of the figure are from the same control person, and those on the *right* from the same hypertensive person. It is evident that, although the vasopressin staining in the SCN of the hypertensive person is diminished compared to the control, CRH staining is enhanced [From Kalsbeek et al. (2010); Fig. 5, with permission]

4.7 Corticosteroids

Impaired sleep and mood disorder are the major side effects of glucocorticoid therapy. The mechanism responsible for the circadian disorder is unknown, but alterations in the SCN are presumed to play a major role. Liu et al. studied the

amount of AVP-mRNA expression in the SCN in 10 glucocorticoid-exposed patients and 10 glucocorticoid-free controls, all well matched for age and clock time of death (Liu et al. 2006). The total amount of AVP-mRNA in the SCN was found to be two times lower in the glucocorticoid-exposed patients than in the control subjects. In addition, there was a 53 % decrease in the total number of profiles in the SCN that expressed AVP-mRNA in glucocorticoid-exposed patients compared with controls (Figs. 4.17 and 4.18). In conclusion, glucocorticoids have an inhibitory effect on AVP-mRNA expression in the human SCN, which may be the biological basis of the circadian rhythm disturbances during glucocorticoid therapy (Liu et al. 2006).

4.8 Conclusion

The human SCN shows functional alterations in relation to day–night, season, age, gender, sexual orientation, tumors compressing the SCN, AD, HD, depression, hypertension, and glucocorticoid exposure. In AD treatment, light and melatonin were found to increase sleep efficiency and to improve nocturnal restlessness, mood, performance, daytime energy, and quality of life.

These data show that functional immunocytochemistry, in situ hybridization, tracing, and morphometrics studies on clinically and neuropathologically well-characterized human postmortem material are not only possible but also meaningful in relation to health and disease.

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Chapter 5
Mammalian Circadian Clocks and Metabolism: Navigating Nutritional Challenges in a Rhythmic World

Jeremy J. Stubblefield and Carla B. Green

Abstract The mammalian circadian clock is vital for generating and coordinating metabolic rhythms. Patterns in feeding behavior present organisms with an influx of nutrients at particular times of day which must be either used or stored. The circadian system helps guide a proper metabolic response to the cellular energy demands by regulating transcriptional and enzymatic activities. Coordination of clocks with metabolic processes is a complex interplay in which both molecular clock machinery and cellular metabolites can influence one another. Genetic disruptions of the circadian system and nutritional perturbations such as restricted feeding or high-fat diet (HFD) consumption have revealed the importance of molecular and metabolic rhythmicity to the health of an organism. In this review, we focus on the responses of the peripheral clocks in mammals to nutritional challenges such as diet and food restriction. Understanding this relationship will help guide the treatment of conditions such as obesity and diabetes.

Keywords Circadian • Obesity • Diabetes • Clock • Restricted feeding

5.1 Molecular Oscillations and Metabolic Challenges

5.1.1 The Circadian Clock

The daily environment presents organisms with a constant flux of energetic challenges. Navigating these challenges and efficiently utilizing energy both in times of

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activity and rest is vital to an organism's survival. Meal timing is of utmost importance in nearly every form of life; so too is the type of food being consumed. Coordinating internal processes with the external environment may seem challenging, but fortunately, most organisms have developed an internal timekeeping system that helps synchronize the energetic needs of their various organ systems.

The internal clock is known as the *circadian system* due to the near 24 h rhythmicity of its own cycling as well as most of the processes that it governs. Behavioral processes such as sleep/wake cycles and feeding are under circadian control. Physiological processes such as body temperature, blood pressure, and nutrient metabolism also occur with a daily rhythmicity. These 24 h rhythms are generated by a molecular oscillator consisting of a series of interlocked transcription/translation feedback loops whereby certain core "clock" genes are transcribed and their protein products build up and inhibit their own transcription (reviewed in Ko and Takahashi 2006). These core rhythms are self-sustained and cell-autonomous and take approximately 24 h to complete, thus making them circadian (about a day). See Chap. 1 for a detailed introduction to the circadian clock.

In mammals, this molecular oscillator exists in nearly every tissue of the body including liver, pancreas, muscle, and lung (Yamazaki et al. 2000; Yoo et al. 2004; Marcheva et al. 2010). The diversity of tissues and organ systems requires some form of synchronization to maintain coordinated rhythmicity. Synchronization is achieved in large part due to the hypothalamic suprachiasmatic nucleus (SCN). Located immediately dorsal to the crossing of the optic nerves at the optic chiasm, the SCN is a binucleated structure whose cells receive direct light information via the retinohypothalamic tract (reviewed in Mohawk and Takahashi 2011). Within the SCN, this light information is the primary *Zeitgeber* (time-giver) serving as an entraining agent to coordinate molecular oscillations with the external light:dark (LD) cycle through induction of immediate early genes, including *Period* 1 (*Per1*) and *Per2*, core components of the clock (reviewed in Chap. 1 and Reppert and Weaver 2002). *Per* feeds into and is part of the core mechanism that generates 24 h rhythms. Within the core oscillator, the transcription of *Per* genes (*Per1*, *Per2*, and *Per3*) and *Cryptochrome* genes (*Cry1* and *Cry2*) occurs via binding of the CLOCK:BMAL1 protein heterodimer to E-box enhancer elements of these genes. NPAS2 is a functional homologue of CLOCK and also regulates transcription by interacting with BMAL1. PER and CRY protein products then accumulate in the cytoplasm as PER:CRY complexes that translocate back into the nucleus and repress the activity of the CLOCK:BMAL1 heterodimer, thus inhibiting their own transcription (Fig. 5.1). Timed PER and CRY protein degradation also contributes to the 24 h molecular rhythm generation (reviewed in Mohawk et al. 2012). CLOCK:BMAL1 also induces *Rev-erb* (α and β) and *ROR* (α and γ) gene expression as part of an interlocking feedback loop that increases the robustness of the core clock machinery. The REV-ERB proteins inhibit, whereas RORα/γ induce expression of *Bmal1* (Preitner et al. 2002; Sato et al. 2004; Akashi and Takumi 2005).

These molecular oscillations within the SCN are vital to rhythmic behavior, as animals with an SCN lesion have arrhythmic sleep, activity, and feeding (reviewed in Moore 2013). The SCN is thus referred to as the "master pacemaker" within

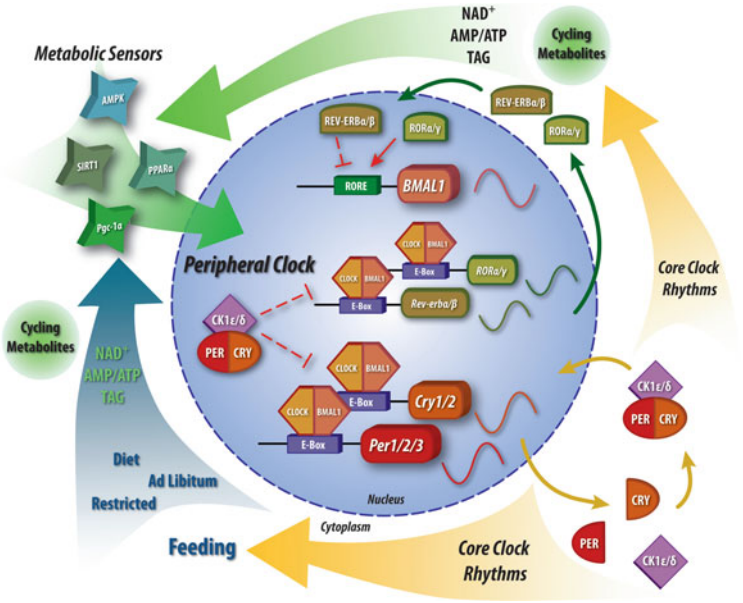

Fig. 5.1 The peripheral clock in mammals drives and responds to rhythms in metabolites. The core clock rhythms in cells of peripheral tissues in mammals are generated by transcriptional activation of the *Period* (*Per 1, 2* and *3*) and *Cryptochrome* (*Cry 1* and *2*) genes by CLOCK:BMAL1 heterodimer binding to E-Box enhancer elements in the *Per* and *Cry* promoters. PER and CRY proteins complex in the cytoplasm (*white*) along with CK1ε/δ and enter the nucleus (*blue*) and inhibit their own transcription through disruption of the CLOCK:BMAL1 complex. CLOCK:BMAL1 also activate transcription of *Rev-erbα/β* and *RORα/γ*. Rhythmic *Bmal1* transcription is regulated through binding of RORα/γ or REV-ERBα/β proteins to RORE elements in its promoter, thus promoting activation or repression, respectively. These two core loops take approximately 24 h to complete and drive rhythms in feeding behavior and cellular metabolites. Cycling metabolites contribute to the molecular oscillations of the clock through proteins acting as metabolic sensors which can respond to changes in the energetic state of the cell such as NAD^+ levels, the AMP/ATP ratio, and levels of triacylglycerol (TAG). The feeding behavior of an organism affects both the type (diet) and timing of nutrient ingestion which can also affect cycling metabolites, thus creating a complex feedback whereby core clock rhythms drive oscillations in cycling metabolites, but can also be influenced by them

mammals, though the core clock machinery exists in peripheral tissues as well. The CLOCK:BMAL heterodimer, in addition to driving the core clock feedback loop, also regulates expression of thousands of genes, many of those involved in metabolism. Thus, the components of this "core" oscillator do not operate independently of the cellular environment in which they reside. In addition to light and body temperature, they receive inputs from molecules such as hormones, nuclear receptors, and nutritional metabolites. These metabolic inputs help shape the resulting rhythms and oscillations (Fig. 5.1). Nutritional challenges impacting the energetic state of an organism are important to study as they provide metabolic input that helps shape molecular rhythms in peripheral tissues. In this review, we will discuss

the molecular oscillations and cycling of nutrient metabolites as well as the physiological responses governed by the circadian system in the context of metabolic or nutritional challenges to help gain a better understanding of the impact of modern lifestyle and dietary situations on overall health.

5.1.2 Diabetes, Obesity, and the Clock

Diabetes and obesity are on the rise worldwide (Chen et al. 2012). While genetic factors certainly contribute to these conditions, increasing evidence for their development through lifestyle and dietary choices leads one to consider the interesting juxtaposition of the circadian clock with metabolism. Jet lag and shift work present interesting cases in which energy intake in the form of feeding become misaligned or occur at inconsistent times in relation to the body's internal clock. The increased consumption of high calorie "western" diets also has links with circadian disruption.

The importance of proper alignment of metabolic processes has been demonstrated through studies of several clock mutant mice (Rudic et al. 2004; Turek et al. 2005; Lamia et al. 2008). The $Clock^{\Delta 19}$ mutant mouse produces a truncated form of the CLOCK protein that can dimerize with its partner BMAL1 and bind to E-boxes but cannot activate transcription and thus acts in a dominant-negative fashion. The $Clock^{\Delta 19}$ mouse gains significantly more weight than wild types when fed a regular chow (RC) diet and becomes significantly more obese than wild-type (WT) littermates when fed a high-fat diet (HFD) (Turek et al. 2005). The increase in body weight on an RC diet is a result of increased energy intake and increased fat mass. Feeding patterns in these mice are altered such that they decrease their evening food intake while increasing the amount consumed during the day. Additionally, these animals have several risk factors associated with the metabolic syndrome including elevated serum levels of glucose, triglyceride, cholesterol, and leptin.

PER2 also has important effects on adiposity in mice. Adult mice with a targeted disruption of the *Per2* gene (Bae et al. 2001) were found to have significantly lower body weight under regular chow conditions that resulted from decreased epididymal white adipose tissue (eWAT) weight and lower total fat mass in whole-body composition analysis (Grimaldi et al. 2010). A critical role for PER2 in adipose tissue was found in this study through interaction between PER2 and the lipid sensor and transcriptional regulator peroxisome proliferator-activated receptor γ2 (PPARγ2). PPARγ2 belongs to the PPAR family of nuclear receptors and is a master regulator of lipid metabolism (Tontonoz and Spiegelman 2008). PER2 was found to inhibit PPARγ2-dependent transcription of key metabolic genes and this resulted in increased adipogenesis in vitro, but enhanced fat oxidation in vivo (Grimaldi et al. 2010). The interaction of PER2 with PPARγ2 occurred independently of CRY proteins and other core clock factors, indicating a unique role for PER2 in regulating fat metabolism outside of its canonical role within the circadian machinery.

The gene *Nocturnin* (*Noc*) encodes a circadian deadenylase whose rhythmic expression is shaped in part by the circadian clock (reviewed in Stubblefield et al. 2012). Deadenylation is a key posttranscriptional process in regulating RNA stability and translatability (Kojima et al. 2011). Nocturnin is expressed in a wide range of tissues with robust rhythmicity in the liver (Wang et al. 2001; Garbarino-Pico et al. 2007) and therefore rhythmic *Noc* expression points toward another mechanistic layer of clock control over metabolism. The core clock contributes to *Noc* expression as *Noc* rhythmicity is dampened in the liver of $Clock^{\Delta 19}$ mice, but *Noc* is also one of the several transcripts that maintain rhythmicity in the liver of animals with an abolished hepatic clock (Oishi et al. 2003; Kornmann et al. 2007). Mice lacking *Nocturnin* ($Noc^{-/-}$) are resistant to diet-induced obesity (DIO) and hepatic steatosis (Green et al. 2007). When placed on a high-fat diet (HFD), $Noc^{-/-}$ mice have altered the rhythmic expression of several key enzymes important for proper lipid metabolism, including *PPARγ* and *Srebp-1c*. The role of the clock in regulating proper *Nocturnin* expression highlights the diversity with which molecular rhythms can contribute to overall physiology.

Obesity is not the only metabolic perturbation seen in clock mutant animals. *Clock*, *Bmal1*, and *Cry1/2* all contribute to proper maintenance of glucose homeostasis (Rudic et al. 2004; Turek et al. 2005; Lamia et al. 2008, 2011; Marcheva et al. 2010; Zhang et al. 2010) and their contributions in this realm will be discussed in greater depth elsewhere in this review (see Sect. 5.3.4). To better understand how the clock (and its disruption) can have such profound metabolic consequences, one must more closely examine the diversity of rhythmic metabolic processes as well as the metabolic inputs that influence clock function.

5.2 Metabolic Rhythmicity

5.2.1 Circadian Output: Omics

CLOCK and BMAL1 are transcription factors with thousands of binding sites within the genome, many of which reside on genes encoding rate-limiting enzymes of key metabolic pathways within the liver (Koike et al. 2012; Le Martelot et al. 2012; Vollmers et al. 2012). Indeed, it is estimated that ~10 % of the hepatic transcriptome is rhythmic (Panda et al. 2002; Storch et al. 2002; Koike et al. 2012; Le Martelot et al. 2012; Menet et al. 2012; Vollmers et al. 2012). Cycling RNA transcripts are only part of the story, however, as posttranscriptional and posttranslational factors also influence molecular rhythms (Kojima et al. 2012). As such, ~20 % of the proteome in liver was found to have circadian rhythmicity, with many of these being enzymes important for vital liver functions, such as urea formation, and energetics, such as carbohydrate metabolism (Reddy et al. 2006; Robles et al. 2014).

Knowing that many rate-limiting metabolic enzymes are cycling both at the RNA and protein level, recent studies have sought to determine the rhythmicity of the liver metabolome and lipidome. Examining different metabolites across the circadian cycle in mice on a regular chow (RC) diet revealed an accumulation of xenobiotic and amino acid metabolites peaking around mid-to-late evening with carbohydrate and lipid metabolites peaking mid-to-late daytime (Eckel-Mahan et al. 2012, 2013; Adamovich et al. 2014). Cycling xenobiotic metabolites is consistent with earlier observations that mRNA transcripts encoding proteins important for detoxification are under clock control (Gachon et al. 2006). Plasma metabolomes have also been examined in mice (Minami et al. 2009) and humans (Kasukawa et al. 2012) with the phasing of the identifiable metabolites being used to approximate internal body time and potentially diagnose circadian rhythm disorders.

Light is the major *Zeitgeber* for molecular oscillations within the SCN, but these rhythms are also self-sustaining. Therefore, removing light and placing animals in constant conditions such as constant darkness (DD) helps reveal a more direct role for the clock in governing behavioral and physiological rhythms. Analysis of the hepatic lipidome in ad libitum (ad lib) fed mice in DD conditions revealed that ~17 % of the identifiable lipids could be classified as having a circadian rhythm (Adamovich et al. 2014). The majority of these cycling lipids were triacylglycerol (TAG) species. Most of the oscillating lipids peaked during the subjective light phase, consistent with the need to liberate stored fat when energy intake is low, in order to maintain metabolic homeostasis. Indeed, gene expression of many of the biosynthetic and catabolic enzymes for TAG metabolism displays circadian rhythmicity in the mouse liver. This same study examined the role of the clock in lipid cycling by performing a similar lipidomic analysis of ad lib fed $Per1^{-/-}/Per2^{-/-}$ mouse liver from DD conditions. Surprisingly, a similar fraction of lipids had significant oscillations and of these, TAG species were again prominent. The peak phasing, however, was nearly opposite to that found in wild-type animals. Thus, lipid oscillation itself may be clock independent, but an intact clock helps dictate the phase.

The temporal aspect of food ingestion is a major behavioral output of the circadian system and this contributes to the cycling of metabolites. Meal timing will be discussed later in this review, but first we will focus on the metabolic sensors both responding to peripheral clocks and informing them of the cellular energetic status as this will help to better understand the molecular links between nutrient metabolism and rhythmic gene expression.

5.2.2 Metabolic Cross Talk

Trying to define circadian inputs becomes difficult as many "inputs" are factors that respond to the metabolic state of an organism, tissue, or cell type. That state is often determined by the temporal energetic condition (fasting vs. feeding), which is in

turn dictated by the clock. Nicotinamide adenine dinucleotide (NAD^+) is a coenzyme in redox reactions and is linked to energetic processes within the cell. NAD^+ synthesis is controlled by the rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT) and *Nampt* gene expression is under clock control (Nakahata et al. 2009; Ramsey et al. 2009). Redox state is important to the core clock mechanism as NAD cofactors can influence CLOCK:BMAL1 and NPAS2:BMAL1 DNA binding (Rutter et al. 2001). There is additional feedback by NAD^+ through the action of SIRT1, an NAD^+-dependent deacetylase which associates with the CLOCK:BMAL1 complex and modulates its function. NAD^+ thus provides a direct link for circadian clock-mediated transcription with the energetic state of a cell (Rutter et al. 2001; Asher et al. 2008; Nakahata et al. 2008). Interestingly, SIRT1 protein and activity both cycle, yet *Sirt1* RNA remains relatively constant. Thus, posttranscriptional mechanisms must contribute to the circadian accumulation of SIRT1 protein. NAD^+ synthesis is under clock control and NAD^+ levels show cycling under ad lib and fasting conditions (Ramsey et al. 2009; Peek et al. 2013); so it would follow that SIRT1 activity would cycle due to its NAD^+ dependence. Not only this is an important feedback cycle for the clock itself, but this rhythmic production of NAD^+ also drives rhythms in mitochondrial function (Peek et al. 2013). Another NAD^+-dependent enzyme, poly (ADP-ribose) polymerase 1 (PARP1), also contributes to proper circadian gene expression. In the liver of mice PARP1 binds to CLOCK:BMAL1 heterodimers and poly(ADP-ribosyl)ates CLOCK which reduces the DNA-binding activity of the heterodimer (Asher et al. 2010). PARP1 not only contributes in this manner to rhythmic transcription, but it also contributes to the phase shifting kinetics of clock genes in the liver when mice are subjected to daytime restricted feeding (Asher et al. 2010).

Metabolic flux within a cell also results in alternating ratios of AMP/ATP. The adenosine monophosphate (AMP)-activated protein kinase (AMPK) can respond to these altered ratios by phosphorylating CRY1 proteins, leading to their destabilization and reduced inhibition of CLOCK:BMAL1 (Lamia et al. 2009). Two isoforms, α1 and α2, exist for the catalytic subunit of AMPK. Mice deficient for the α1 isoform ($Ampk\alpha1^{-/-}$) have specific deficits in circadian behavior; however, $Ampk\alpha2^{-/-}$ mice do not (Um et al. 2011). The α1 isoform is expressed predominantly in adipose and brain, whereas the α2 isoform is predominant in muscle. Loss of each isoform individually has differential effects on clock-gene expression in a tissue-specific manner. While overt behavioral rhythmicity is maintained in both the $Ampk\alpha1^{-/-}$ and $Ampk\alpha2^{-/-}$ mice, the impairments that $Ampk\alpha1^{-/-}$ mice exhibit help support a role for AMPK in shaping the molecular oscillations of the core clock in vivo.

Recently, another molecular link between glucose metabolism and core clock components was revealed. The protein *O*-GlcNAc transferase (OGT), which mediates protein glycosylation, was found to contribute to both CLOCK:BMAL1 transcriptional activity and posttranslational modification of PER2 (Kaasik et al. 2013; Li et al. 2013). Glycosylation catalyzed by OGT utilizes UDP-GlcNAc, a by-product of glucose metabolism via the hexosamine pathway.

Overexpression of OGT was found to stabilize CLOCK proteins while decreasing ubiquitination of both CLOCK and BMAL1, leading to increased expression of their target genes *Per2* and *Cry1* in vitro (Li et al. 2013). *O*-GlcNAcylation also competes with phosphorylation of human PER2 protein on serine residues whose regulation is important for overt circadian behavior, as their mutation results in familial advanced sleep-phase disorder (Toh et al. 2001; Kaasik et al. 2013). Mice with a liver-specific loss of OGT have disruptions in plasma glucose rhythmicity and overall glucose metabolism (Li et al. 2013). OGT activity cycles and is regulated in part by glycogen synthase kinase 3β (GSK3β) (Kaasik et al. 2013). This regulation is important for proper behavioral rhythmicity as inhibition of GSK3β in mice alters circadian wheel-running activity (Kaasik et al. 2013). Taken together, these findings further expand the role of metabolic sensors in fine-tuning clock function.

5.2.3 Nuclear Receptors Impact the Clock

Nuclear receptors (NRs) are metabolic sensors that regulate the transcription of a variety of genes in a ligand-dependent manner. These ligands are composed mainly of hormones and nutrient metabolites. Transcripts under their control include key players in metabolic signaling cascades. Many nuclear receptors show clear patterns of rhythmic expression globally and in a tissue-specific manner (Yang et al. 2006). Among them are two components of the core circadian feedback loop REV-ERBα and RORα. As previously described, these two orphan nuclear receptors contribute to proper *Bmal1* transcription. A *Rev-erbβ* isoform also exists and has been shown to repress *Bmal1* transcription as well (Guillaumond et al. 2005). *Rev-erbα*$^{-/-}$ mice exhibit a significantly shorter free-running period in constant darkness, though *Rev-erbβ*$^{-/-}$ do not show significant alterations. A recent report utilizing an inducible cre/lox system found that inducible double knockout of both *Rev-erbα* and *Rev-erbβ* (iDKO) in adult mice resulted in a free-running period shorter even than that seen in *Rev-erbα*$^{-/-}$ mice alone. Additionally, these iDKO animals displayed fasting hypoglycemia and hypotriglyceridemia (Cho et al. 2012). When the double knockout of *Rev-erbα* and *Rev-erbβ* was restricted to the liver using the albumin-cre driver, this significantly disrupted the cycling of many genes associated with key metabolic pathways such as insulin signaling and the tricarboxylic acid (TCA) cycle (Cho et al. 2012). The nuclear receptor coactivator PGC-1α provides an additional layer of metabolic regulation of peripheral clock function by regulating *Bmal1* and *Rev-erbα* expression through coactivation of the ROR family of nuclear receptors (Liu et al. 2007).

The peroxisome proliferator-activated receptor α (PPARα) also forms a significant metabolic feedback loop with the core clock machinery. PPARα was shown to directly bind to PPAR response elements in the promoters of both *Bmal1* and *Rev-erbα* (Canaple et al. 2006). The PPAR family of nuclear receptors are important lipid sensors that transcriptionally regulate lipid processing through utilization and

storage (Evans et al. 2004). PPARα gene expression is regulated by the circadian clock and cycles in vivo (Oishi et al. 2005). Several different PPAR isoforms exist (PPARα, PPARγ, and PPARδ) with tissue-specific expression. As mentioned previously, Nocturnin has also been shown to regulate proper nuclear receptor functioning through its regulation of hepatic *PPARγ* expression under HFD conditions (Green et al. 2007). It has also been shown to participate in nuclear shuttling of PPARγ in vitro in 3T3-L1 cells (Kawai et al. 2010).

The retinoic acid receptor α (RARα) and retinoid x receptor α (RXRα) exist in complexes that respond to retinoic acid as a ligand and *RARα* mRNA cycles in multiple tissues (Yang et al. 2006). In the presence of their hormonal ligand, both RARα and RXRα associate with CLOCK and NPAS2 and reduce transcriptional activation by CLOCK:BMAL1 and NPAS2:BMAL1 (McNamara et al. 2001). A screen looking for potential entraining agents in rat fibroblasts stably transfected with an *mPer2*-luc reporter revealed that several forms of retinoic acid can significantly affect rhythm entrainment (Nakahata et al. 2006). Additionally, administration of all-*trans*-retinoic acid in mice significantly phase-shifts *Per2* rhythms in the heart (McNamara et al. 2001). RXRα also forms a heterodimer with PPARγ and thus contributes to the transcriptional program of lipid metabolism (Tontonoz and Spiegelman 2008).

Corticosterone is the major murine glucocorticoid and this hormone cycles with robust rhythmicity. While expressed in most metabolic tissues, the glucocorticoid receptor (GR) is only rhythmic in white and brown adipose tissue under ad lib RC conditions (Yang et al. 2006). Glucocorticoids are used as entraining agents in cultured cells due to their induction of clock gene transcripts and they elicit phase shifts in clock gene expression in peripheral tissues upon injection (Balsalobre et al. 2000a, b). However, they were also found to inhibit the restricted-feeding phase shifting of clock gene expression in peripheral tissues in a GR-dependent manner (Le Minh et al. 2001). Corticosterone is secreted rhythmically by the adrenal gland and adrenalectomized mice displayed much faster phase resetting of clock gene expression in liver and kidney (Le Minh et al. 2001). While glucocorticoids may play important roles in tissue synchronization under ad lib conditions, they do not appear to be the primary *Zeitgeber* for peripheral tissues under restricted feeding conditions. In a restricted feeding paradigm, the liver receives nutrient signals dictated by the feeding time, yet signals such as glucocorticoids are still being driven in part by the master pacemaker within the SCN. Thus, glucocorticoid interference in peripheral clock shifting highlights the opposing actions of the central clock in the SCN and the peripheral clocks in response to altered feeding regimens. Still, CRY1 associates with the GR and represses glucocorticoid-induced transcriptional activation, meaning that the core clock components contribute to glucocorticoid regulation of metabolic processes, if not entrainment (Lamia et al. 2011).

5.3 Feeding Cycles and Diet Influence Peripheral Clocks

5.3.1 Feeding: When You Eat Matters

For animals in the wild, meal timing is often more restricted than the ad lib conditions of the laboratory setting. Providing the mice with free access to food has allowed researchers to examine the roles of light:dark (LD) cycles, constant conditions such as constant dark (DD) and constant light (LL), as well as the clock itself on feeding behavior. Understanding the role of the clock in feeding requires consideration of both central and peripheral clocks. Contributions of the central clock to feeding centers in the brain have been reviewed elsewhere (Marcheva et al. 2013) and we will instead focus here on the effects of particular feeding regimens on peripheral clocks.

Feeding is an important entraining agent for peripheral circadian clocks, especially the liver. When either rats or mice experience temporal restricted feeding, where their access to food is limited to a few hours during the light phase, clock gene expression shifts in peripheral tissues (Damiola et al. 2000; Stokkan et al. 2001). The kinetics of this shift are tissue specific as the liver shifts fairly rapidly compared with kidney, heart, and pancreas (Damiola et al. 2000). Of note, clock gene expression within the SCN remains unaffected by the restricted feeding, implicating a greater importance of nutrient signals in the entrainment of oscillators in the periphery and an uncoupling of the central pacemaker with peripheral tissues in altered feeding conditions.

While restricted feeding can uncouple the central and peripheral clocks, determining the role of the master pacemaker in peripheral entrainment under ad lib conditions can be difficult. As light is the major *Zeitgeber* for the master pacemaker in the SCN, removal of light as an input by placing animals in DD has been used to assess the autonomous nature of the clock in behavioral and physiological rhythms. As previously mentioned, animals put in DD were found to have oscillations in a large number of liver mRNAs and proteins. Mice in DD maintain rhythmic feeding, however, and so the cycling of many of these transcripts could still be a result of the feeding rhythm and not directly the local peripheral clock.

To help tease apart the different contributions of food vs. the clock, Vollmers and colleagues examined transcriptional changes in the livers of both wild-type and $Cry1^{-/-}/Cry2^{-/-}$ mice in response to fasting, refeeding, and temporal restricted feeding (Vollmers et al. 2009). Food availability for only 8 h during the light phase was able to shift expression of many of the transcriptional targets of key metabolic regulators such as CREB, AKT, SREBP1/2, and ATF6. While many of these transcripts were arrhythmic under ad lib feeding in $Cry1^{-/-}/Cry2^{-/-}$ livers, they gained rhythmicity following the temporal restricted feeding. If food was removed altogether and hepatic gene expression assessed during 24 h of fasting, it was found that more than 80 % of the genes found to cycle under ad lib conditions ceased to oscillate. A small percentage maintained rhythmicity, and contained within this group were the components of the core clock. This is yet further evidence that

rhythms in peripheral transcriptomes are heavily influenced by the metabolic state of the organism, namely the timing of food ingestion.

Analysis of transcriptomes and proteomes is only part of the metabolic landscape. Determining actual metabolite levels in various tissues will help fill out the picture of how the clock and feeding cycles impact overt physiology. As previously mentioned, Asher and colleagues conducted a comprehensive analysis of the hepatic lipidome in wild-type and $Per1^{-/-}/Per2^{-/-}$ mice fed either ad lib or restricted to the dark phase (Adamovich et al. 2014). Their findings showed that a substantial fraction of lipids cycled in both WT and $Per1^{-/-}/Per2^{-/-}$ mice under ad lib conditions (~17 %), yet the oscillating species in $Per1^{-/-}/Per2^{-/-}$ were mostly distinct from those in WT. While total daily food intake did not differ between the genotypes, $Per1^{-/-}/Per2^{-/-}$ mice lost their temporal consolidation of food intake to the dark phase and instead ate relatively equal amounts throughout the day. This unique oscillating population of lipids in ad lib $Per1^{-/-}/Per2^{-/-}$ mice thus represents a fraction of lipids whose control is not mediated entirely by the feeding pattern or the clock. A clear mechanism for their regulation is an area open for investigation. Temporal food restriction to the night phase altered the phasing of TAG accumulation with peaks around CT12 in both genotypes, corresponding with the presentation of food. Surprisingly, the oscillating TAG species now showed a clear overlap between WT and $Per1^{-/-}/Per2^{-/-}$ animals, indicating that meal timing can dictate the phasing of certain TAGs in the absence of a functional clock. Another important finding was that feeding restricted to the night phase lowered the total hepatic TAG levels by ~50 % in WT animals compared to ad lib feeding. This reduction in lipid content of the liver is consistent with other recent findings that temporal restriction of food intake can improve metabolic parameters such as hepatic steatosis (Hatori et al. 2012). Taken together, these data show that both the clock and feeding patterns can have significant impacts on both transcript and metabolic cycling, with important impacts on overall physiology, while also impacting one another.

5.3.2 Diet: What You Eat Matters

While the timing of nutrient intake is important, the type of nutrients taken in during feeding directly influences the clock as well. HFD feeding can change not only behavioral rhythms, but also molecular and metabolite oscillations (Kohsaka et al. 2007; Pendergast et al. 2013; Adamovich et al. 2014). As mice are progressively exposed to a HFD (containing 45 % kcal from fat), their daytime locomotor activity increases, as does their food consumption during the light phase (Kohsaka et al. 2007). This is accompanied by alterations in clock gene expression in both hepatic and adipose tissue with a general trend toward damping of oscillations. Transcript levels of many key metabolic regulators of fat metabolism also display altered patterns with tissue-specific effects. Additionally, circulating hormones that

normally oscillate under an RC diet are altered with HFD, though hypothalamic clock gene expression remains largely unaffected.

If one looks broadly at the effects of diet on the liver transcriptome and metabolome, a significant reorganization of molecular oscillations by high fat energy consumption is seen. Ten weeks of HFD consumption (60 % kcal from fat) results in obesity, hepatic steatosis, hypertriglyceridemia, and impaired glucose tolerance (Eckel-Mahan et al. 2013). After HFD feeding, there was a significant change in cycling metabolites such that some which were previously rhythmic lost their cycling and others which previously did not cycle became rhythmic, while some remained unchanged. A similar alteration was seen in cycling transcripts in the liver. The circadian clock genes remained rhythmic in RC and HFD conditions, again highlighting the importance of nutrient and feeding cues on peripheral clocks. While the clock genes maintaining rhythmicity, HFD was found to alter the ability of CLOCK:BMAL1 to bind to target gene promoters. These effects of diet began to manifest with only 3 days of HFD exposure in mice and, remarkably, began to reverse when HFD animals were shifted back to RC feeding for 2 weeks.

The type and timing of food consumption both have profound effects independently on the circadian clock and rhythmic metabolic profile. It should not be surprising then that the combined effects of these two metabolic signals could have important effects on energy metabolism. Mice with time-restricted feeding (tRF) of a HFD to the night phase were found to be protected from DIO and many of the negative effects of ad lib HFD feeding. These animals had improvements in factors important for glucose metabolism (including phosphorylated cAMP response element-binding protein (CREB) and hepatic AMP levels), as well as fat oxidation (including phosphorylated acetyl-CoA carboxylase), compared to their ad lib fed HFD counterparts (Hatori et al. 2012). The tRF mice that were fed HFD only during the night were better able to utilize fat as an energy source as reflected in their improved respiratory exchange ratio and energy expenditure. Both hepatic steatosis and glucose metabolism were improved in the tRF HFD animals as well. While tRF in RC animals did not improve metabolic processes to the same extent, the marked improvements in the HFD animals can be an important guide in studying the temporal consequences of feeding.

5.3.3 Nutrient Uptake

When considering the temporal aspect of food consumption, nutrient uptake must also be considered. When fed ad lib, mice consume around 70 % of their daily caloric intake in the evening hours (Kohsaka et al. 2007; Adamovich et al. 2014). Indeed, even though feeding behavior per se appears under the control of the circadian system, the uptake of the resulting nutrients is also gated by the clock. Due to the near ubiquitous nature of clock genes in different cell types, it is not surprising that the core clock machinery exists in cells of both the small intestine and colon (Hoogerwerf et al. 2007; Pan and Hussain 2009). Clock gene rhythmicity

in the colon persists in both the absence of LD cycles (in DD) and in vagotomized mice (Hoogerwerf et al. 2007), indicating a functional, local clock within the digestive tract. The small intestine is the major site of nutrient absorption and investigations into the temporal efficiency of nutrient absorption revealed that carbohydrates, peptides, lipids, and cholesterol all show a time-dependent variance in their uptake (Pan and Hussain 2007, 2009). Absorption was higher during the middle of the dark phase compared to those during the middle of the light phase. This can likely be attributed to the rhythmic expression of many of the key transporters of these nutrients within the intestine (Pan et al. 2002, 2004; Pan and Hussain 2007). It is still not clear whether the clock components directly regulate the expression of all of these transporters, as they too respond to different feeding regimens by altering their expression. Still, it remains clear that the intestine is primed to optimally absorb nutrients at certain times of day.

To help address the role of the clock, Pan and Hussain also investigated $Clock^{\Delta 19}$ mutant mice, which develop obesity, and found them to have equal absorption of nutrients during both the day and night hours, consistent with the loss of temporal partitioning of their food intake (Turek et al. 2005; Pan and Hussain 2009). The $Clock^{\Delta 19}$ mutant mice used in this study were whole-body mutants. It will be interesting to examine the effects of the local clock on nutrient absorption using the more selective conditional knockout animals that are now available. Investigations into complete loss of *Clock* in $Clock^{-/-}$ mice, which do not show the same severity of metabolic abnormalities with HFD as the $Clock^{\Delta 19}$ model, have disrupted fat absorption (Oishi et al. 2006). This disruption is thought to occur through improper lipid digestion in the stomach due to reduced daytime release of lipase. It should also be noted that the $Clock^{\Delta 19}$ and $Clock^{-/-}$ mice in each of these studies were on different genetic backgrounds (Turek et al. 2005; Oishi et al. 2006). Mice deficient for the rhythmically expressed deadenylase Nocturnin ($Noc^{-/-}$) also show deficits in lipid and cholesterol flux through intestinal enterocytes (Douris et al. 2011). This helps explain their resistance to DIO (Green et al. 2007), though a specific mechanism remains to be elucidated.

5.3.4 Glucose and Insulin Homeostasis

The temporal pattern of feeding results in a majority of caloric intake at certain times of day. Thus, nutrients taken in and not immediately used are stored for utilization during the intervening periods in order to maintain metabolic homeostasis. During times of fasting, the liver is the main site of glucose and ketone body production via gluconeogenesis (reviewed in Previs et al. 2009) and ketogenesis (reviewed in Cotter et al. 2013), respectively. Fasting is an important process that itself is influenced by the circadian system. In mammals, blood glucose is a critical parameter that must be maintained within strict margins (euglycemia), even when energy intake is restricted. Blood glucose values cycle throughout the day in mammals and proper circadian alignment contributes to this rhythmicity (Scheer

et al. 2009). Importance of maintaining proper glucose rhythmicity is evidenced indiabetic patients who often lose this control (Polonsky et al. 1988). Additionally, genetic disruption of clock components leads to altered ad lib and fasting glycemia (Turek et al. 2005; Lamia et al. 2008).

Since meal timing can vary throughout the day, organisms must contend with large influxes of nutrients and maintain euglycemia. A normal physiological response to a bolus of glucose is for the pancreas to secrete insulin which signals to the liver for a shutdown of gluconeogenesis and ketogenesis as well as to other peripheral tissues to take up glucose from the blood. One way of assessing insulin sensitivity is the glucose tolerance test, in which a bolus of glucose is administered and an animal's response as measured by blood sugar levels indicates their ability to regulate glucose homeostasis (Ayala et al. 2010). Clock mutant animals have illuminated a critical role for the circadian clock in mediating these physiological responses (Table 5.1). $Clock^{\Delta 19}$ animals have altered glucose metabolism as displayed by hyperglycemia in the ad lib state (Turek et al. 2005). The $Clock^{\Delta 19}$ and $Bmal1^{-/-}$ mice have altered plasma glucose responses to an injection of insulin during the day and night (Rudic et al. 2004). Additionally, animals consuming an RC diet display a trend toward decreased glucose tolerance in the early morning which is exacerbated by HFD consumption (Rudic et al. 2004). A similar study found the day–night difference in glucose tolerance of animals fed a HFD to be somewhat blunted (Prasai et al. 2013), though glucose dose, age, and dietary fat content were somewhat different than the experiments conducted by Rudic and colleagues, and these factors have been shown to critically influence glucose disposal (Ayala et al. 2010). Chronic feeding of a HFD leads to decreased daytime glucose tolerance, but remarkably, if the HFD feeding is restricted to 8 h during the dark phase, daytime glucose tolerance returns to levels seen in regular chow, ad lib fed animals (Hatori et al. 2012). Surprisingly, the $Noc^{-/-}$ mouse which is resistant to DIO has reduced daytime glucose tolerance but increased insulin sensitivity under RC conditions (Green et al. 2007). Interestingly, HFD feeding produces insulin resistance in WT and $Noc^{-/-}$ mice, thus making the $Noc^{-/-}$ mouse an interesting model for studying the effect of diet on a diabetic phenotype independent of obesity.

A closer examination of glucose metabolism in $Clock^{\Delta 19}$ and $Bmal1^{-/-}$ revealed an important role for the circadian clock in regulating pancreatic function. Importantly, the pancreas contains a functional clock with oscillations of core clock genes in vivo and *Per2-Luc* in vitro (Marcheva et al. 2010). The hyperglycemia reported in $Clock^{\Delta 19}$ animals is due to elevated blood glucose during both the light and dark phases and this is accompanied by decreased insulin levels in the dark phase (Marcheva et al. 2010). Probing glucose tolerance, $Clock^{\Delta 19}$ animals had a subtle phenotype of reduced glucose tolerance, again accompanied with reduced insulin levels in response to an intraperitoneal glucose injection. Studying isolated pancreatic islets from $Clock^{\Delta 19}$ animals revealed that these clock mutants have decreased insulin release as a result of reduced insulin exocytosis. This defect is not limited to the $Clock^{\Delta 19}$ mutant, as $Bmal1^{-/-}$ mice display similar pancreatic deficits. Importantly, the local pancreatic clock is vital to proper pancreatic function as

Table 5.1 Glucose tolerance test phenotypes in wild-type and circadian mutant mice

Gene	Loss or gain of function	Location	Genetic background	Diet	Time	Fast duration	Glucose dose	Phenotype	References
Wild type	N/A	N/A	Unspecified	RC	CT1 vs. CT13	12 h	0.1 g/kg (IP)	Daytime response trending toward intolerance, but not statistically significant	Rudic et al. (2004)
			Unspecified	HFD (Teklad, TD02435)	CT1 vs. CT13	12 h	0.1 g/kg (IP)	Elevated fasting glucose at CT1 with daytime glucose intolerance	
			C57BL/6	RC	ZT1 vs. ZT13	12 h	1 g/kg (IP)	No significant difference in overall response	Prasai et al. (2013)
			C57BL/6	HFD (Bio-serv 60 % kcal from fat)	ZT1 vs. ZT13	12 h	1 g/kg (IP)	No significant difference in overall response	
Bmal1	*Bmal1*$^{-/-}$	Whole body	C57BL/6 × 129	RC	ZT4.5	2 h	2 g/kg (IP)	Severe glucose intolerance	Lamia et al. (2008)
	L-Bmal1$^{-/-}$	Liver-specific	C57BL/6 × 129	RC	ZT4.5	2 h	2 g/kg (IP)	Improved glucose tolerance	
			C57BL/6 × 129	RC	ZT4.5	Overnight	2 g/kg (IP)	Improved glucose tolerance	
	PdxCre Bmal1$^{flx/flx}$	Pancreas-specific	C57BL/6	RC	ZT2	14 h	2 g/kg (IP)	Severe glucose intolerance	Marcheva et al. (2010)
Clock	*Clock*$^{\Delta 19}$	Whole body	C57BL/6	RC	ZT14	Overnight	2 g/kg (IP)	Moderate glucose intolerance	Marcheva et al. (2010)

(continued)

Table 5.1 (continued)

Gene	Loss or gain of function	Location	Genetic background	Diet	Time	Fast duration	Glucose dose	Phenotype	References
Cry	*Cry1*$^{-/-}$	Whole body	C57BL/6 × 129	RC	Unspecified, Daytime	Overnight	2 g/kg (IP)	Severe glucose intolerance	Lamia et al. (2011)
	Cry2$^{-/-}$	Whole body	C57BL/6 × 129	RC	Unspecified, Daytime	Overnight	2 g/kg (IP)	Moderate glucose intolerance	
	Cry1$^{-/-}$/*Cry2*$^{-/-}$	Whole body	C57BL/6 × 129	RC	Unspecified, Daytime	Overnight	2 g/kg (IP)	Severe glucose intolerance	
	Ad-Cry1 (Cry1 overexpression)	Liver-specific	*db*/*db*	RC	Unspecified, Daytime	Overnight	1 g/kg (IP)	Improved glucose tolerance	Zhang et al. (2010)
Nocturnin	*Nocturnin*$^{-/-}$	Whole body	C57BL/6	RC	ZT4	16 h	1.5 g/kg (IP)	Moderate glucose intolerance	Green et al. (2007)

RC regular chow, *HFD* high-fat diet, *CT* circadian time refers to animals kept in constant conditions, in this case constant darkness with CT0 defined as the time of activity offset and CT12 time of activity onset; *ZT* Zeitgeiber Time refers to animals kept in light:dark cycles with ZT0 defined as time of lights ON and ZT12 defined as time of lights OFF; *IP* intraperitoneal

PdxCreBmal1$^{flx/flx}$ mice, which have a pancreas-specific clock disruption, recapitulate the phenotypes seen in the whole-body *Bmal1*$^{-/-}$ mutants with decreased glucose tolerance and reduced insulin exocytosis.

The glucose phenotype seen in whole-body *Bmal1*$^{-/-}$ mutants is due, at least in part, to loss of *Bmal1* in the liver, since conditional knockout of *Bmal1* specifically in the liver (*L-Bmal1*$^{-/-}$) resulted in disrupted cycling of key regulatory genes important for glucose homeostasis (Lamia et al. 2008). Some of these genes retained their rhythmicity, though with altered phasing of expression. Of note, the major hepatic glucose transporter *Glut2* was expressed at very low levels and no longer cycled at either the RNA or protein level in *L-Bmal1*$^{-/-}$ mice. Additionally, these *L-Bmal*$^{-/-}$ mice displayed hypoglycemia only during the light phase of activity (when food intake is reduced) and a similar, though blunted response to insulin. These results, combined with those discussed above from pancreas-specific *Bmal1*$^{-/-}$ knockout (Marcheva et al. 2010), highlight the complex interplay of clocks within different peripheral tissues.

CREB is an important mediator of the gluconeogenic program (Altarejos and Montminy 2011) and its activity-dependent phosphorylation was found to cycle (Vollmers et al. 2009). During fasting, gluconeogenesis is high and this is regulated in part through glucagon stimulation of CREB (Altarejos and Montminy 2011). Administration of glucagon induced the key gluconeogenic genes *G6pc* and *Pck1* to a greater extent in the early part of the dark phase as compared to the early part of the light phase following a fast (Zhang et al. 2010). This induction was significantly reduced in mice with adenoviral overexpression of CRY1 and these mice also had fasting-induced hypoglycemia. Conversely, RNAi-mediated knockdown of hepatic *Cry1* and *Cry2* in vivo resulted in increased mRNA expression of *G6pc*, the gene that encodes the catalytic subunit of glucose-6-phosphate, and *Pck1*, which encodes the cytosolic form of phosphoenolpyruvate carboxykinase. Importantly, adenoviral overexpression of CRY1 in the genetically obese *db*/*db* mice significantly improved glucose tolerance. Loss of either *Cry1* or *Cry2* results in significant loss of glucose tolerance during the daytime. Combined loss of *Cry1* and *Cry2* in *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice results in an even more severe loss of glucose tolerance during the light phase (Lamia et al. 2011). This is thought to act through increased corticosterone production (an important mediator of gluconeogenesis) and loss of CRY interaction with the GR, resulting in increased *Pck1* expression. Together, these loss- and gain-of-function studies provide compelling evidence for the importance of the circadian clock in regulation of glucose homeostasis.

5.4 Conclusions and Final Perspectives

The circadian clock is vital to coordinating metabolic rhythms in mammals and disruptions to the circadian system can have profound impacts on health, specifically glucose and lipid metabolism. The studies highlighted here provide evidence of the complex interplay between the molecular clock machinery and metabolites.

A metabolic loop exists in which metabolites and clocks can influence one another. Genetic disruptions of the clock can disrupt behavioral processes such as feeding behavior, but equally as disruptive is the simple ad lib consumption of a HFD diet. Restricting food intake temporally can have beneficial effects on metabolism, but understanding these effects requires a dissection of the clock in conditions of nutritional challenge. Recent studies have begun probing these metabolic perturbations in detail and we have tried to synthesize many of those findings here. Moving forward, understanding the relationship between the circadian system and metabolism will help guide the treatment of conditions such as obesity and diabetes.

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Chapter 6
Circadian Regulation of Renal Function

Kristen Solocinski, Gianluigi Mazzoccoli, and Michelle L. Gumz

Abstract The biological clock allows living organisms to anticipate periodic changes in the external environment and this feature allows a competitive advantage at both the species and individual level. Among the physiological parameters which need accurate adjustment during a 24-h period are fluid, electrolyte, acid–base balance, urine production, and maintenance of blood pressure. These functions are all mediated by the kidneys—organs that are critical for the regulation of blood pressure and the maintenance of body homeostasis. Developing evidence clearly demonstrates a role for the molecular circadian clock in the regulation of several renal ion transporters and channels with implications for circadian control of renal function.

Keywords Ion transport • Electrolyte • Clock • Hypertension • Sodium

6.1 Introduction

6.1.1 Physiological Functions and Structure of the Kidney

The kidney is the organ primarily responsible for maintaining blood volume, pH balance, and electrolyte homeostasis. The kidney is a complex organ that filters the blood and thus plays a critical role in determining the body fluid composition and blood pressure. Production of urine and excretion of metabolic waste products from the blood are critical renal functions as well. Blood plasma is filtered through the

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glomerulus, an intricate structure consisting of several distinct cell types that together form a filtration unit (the term "glomerulus" is named from the Latin word *glomus*, meaning a ball of yarn) [for an excellent review on this structure, see Scott and Quaggin (2015)]. Each glomerulus is attached to a segmented tubule to form a nephron, the functional unit of the kidney. Glomeruli generate the primary urinary filtrate which then passes through the epithelial tubule, first into the proximal tubule (PT), then to the loop of Henle, the distal convoluted tubule (DCT), and finally into the collecting duct (CD). The CDs empty into the ureter to eventually reach the urinary bladder. A typical human kidney consists of about 1 million nephrons. Each day the kidneys filter approximately 200 l of fluid! The kidney ultimately concentrates this volume into ~1–2 l of urine.

The kidney consists of an outer layer, designated the cortex, which contains the glomeruli, proximal tubules, distal convoluted tubules, and cortical collecting duct (CCD) (Fig. 6.1). The inner region of the kidney is divided into the outer and inner medulla wherein the loops of Henle as well as the outer medullary CD (OMCD) and inner medullary CD (IMCD) are located. The nephron is a hollow tubule composed of several discrete segments each with a single layer of epithelial cells.

Specialized cell types with distinct functions are located along each nephron segment. Through the function of these heterogeneous cell types, the kidney carefully maintains the pH and electrolyte balance. The polarized epithelial cells of the nephron tightly regulate the movement of water (H_2O), ions, including sodium (Na) and potassium (K), and organic solutes such as glucose into and out of the filtrate that passes through the nephron. Figure 6.1 illustrates three such cell types. The apical membrane of each cell type faces the lumen of the tubule, whereas the basolateral membrane faces the renal interstitium leading to the capillary network of the renal vasculature. Common to all cell types is the Na/K-ATPase which is localized to the basolateral membrane where it mediates sodium ion entry back to the blood in exchange for potassium ions. The proximal tubule is responsible for the reabsorption of roughly 67 % of the sodium that is filtered at the glomerulus. In cells of the proximal tubule (Fig. 6.1A), an apical sodium/hydrogen exchanger (NHE3) mediates sodium entry into the cell in exchange for hydrogen ions that acidify the filtrate. Sodium/glucose symporters (SGLT1 is pictured) are also present in this cell type. About 25 % of the filtered sodium load is reabsorbed in the loop of Henle and this segment contributes to the countercurrent mechanism that drives concentration of the urine (Fig. 6.1B) (Koulouridis and Koulouridis 2014). The DCT (Fig. 6.1C) is responsible for approximately 4 % of sodium reabsorption along the nephron and this occurs through the action of the sodium chloride cotransporter (NCC) located on the apical membrane of cells in this segment. The renal epithelial sodium channel (ENaC) is located on the apical membrane of principal cells in the collecting duct (Fig. 6.1D) where it mediates sodium entry into these cells. Also present in the CD are intercalated cells that express several transport proteins that regulate acid/base and pH balance. Although the distal nephron mediates a small percentage of total sodium reabsorption, the DCT and CD are the site of very fine-tuned hormonal regulation, enabling the kidney to adapt quickly to changes in electrolyte balance in order to maintain

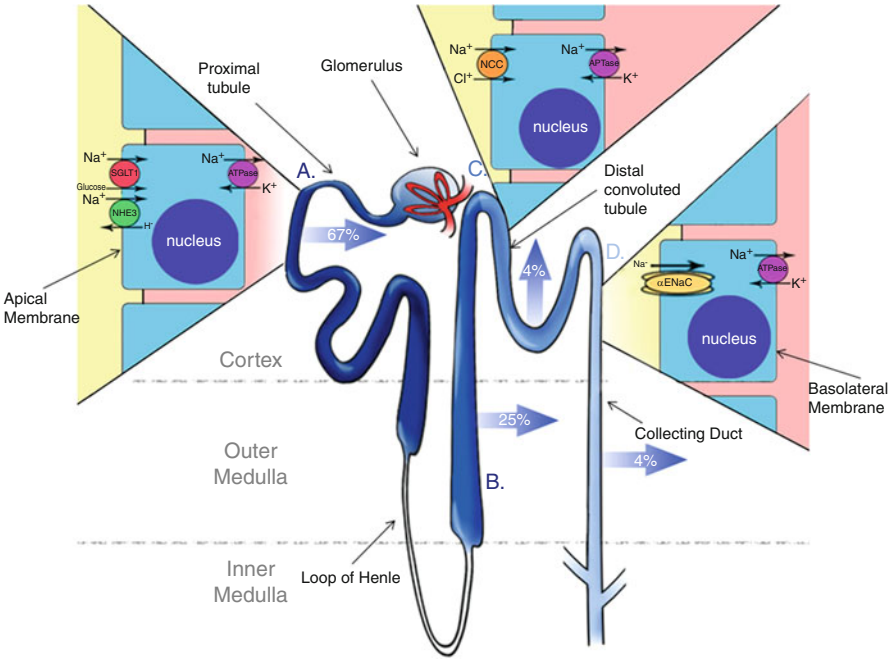

Fig. 6.1 A Nephron and Representative Renal Cell Types. Blood enters the nephron and is filtered at the glomerulus. Filtrate then moves to the proximal tubule (PT). (A) Cells in the PT of the nephron contain the sodium–glucose linked transporter isoform 1 (SGLT1) and the sodium–hydrogen exchanger isoform 3 (NHE3), both of which are located on the apical membrane (facing the filtrate) and are regulated by the circadian clock. Sodium reabsorbed from the filtrate is pumped back into the blood by the basolateral Na/K-ATPase, found on each of the cell types illustrated here. (B). Filtrate then moves to the loop of Henle. (C) The distal convoluted tubule cells contain apically located sodium chloride cotransporters (NCC) which reabsorb Na^+ and Cl^- from the filtrate. This cotransporter is regulated by the circadian clock. (D) After passing through the distal nephron, the filtrate enters the collecting duct (CD). The epithelial sodium channel (ENaC) reabsorbs Na^+ from the filtrate and is regulated by the clock. Glomeruli, PTs, and DCTs are located in the cortex of the kidney, while the loop of Henle and part of the collecting duct span into the medulla. Multiple collecting ducts converge into the ureter, emptying the filtrate into the bladder to become the urine. Figure adapted from (Solocinski et al. 2015)

homeostasis. Circadian clock-mediated regulation of renal function is perhaps best characterized in terms of sodium transport mechanisms. Each of the proteins pictured in Fig. 6.1 has been linked to the molecular clock. For a review of circadian rhythms in potassium homeostasis, see Gumz and Rabinowitz (2013). Circadian regulation of sodium transport mechanisms in the three cell types discussed above will be presented in Sect. 6.3.

6.1.2 The Molecular Kidney Clock

As introduced in Chap. 1, the molecular clock mechanism involves a transcription translation feedback loop made up of several core circadian clock proteins that function as transcription factors to regulate gene expression. In the positive loop, the core proteins BMAL1 and CLOCK heterodimerize and target E-box response elements in the promoters of the Period and Cryptochrome genes, *Per* and *Cry*. In the negative loop, PER and CRY feedback on and interfere with transcriptional activation by BMAL1:CLOCK, thereby decreasing their own transcription. There are several isoforms of the core clock genes, including *Clock* and its homologue *Npas2*, as well as *Per1*, *2*, and *3* and *Cry1* and *2*. Several layers of regulation are involved in maintaining the clock mechanism and these include negative regulation of *Bmal1* expression by REV-ERBα (encoded by the gene *Nr1d1*, hereafter referred to as *Reverba*) and positive regulation of *Bmal1* by RORα. Posttranscriptional and posttranslational mechanisms contribute as well (see Chap. 1 for a detailed consideration of the interlocking loops and intricate mechanism of the molecular clock). For instance, PER1 must be phosphorylated by casein kinase 1δ/ε (CK1δ/ε) in order to enter the nucleus (Badura et al. 2007; Lee et al. 2011).

A landmark transcriptomics study aimed at characterizing circadian gene regulation in multiple tissues revealed that nearly 50 % of all expressed genes throughout the body are controlled by the clock mechanism, in a tissue-specific manner (Zhang et al. 2014). Importantly, this study demonstrated that the kidney was second only to the liver in terms of the absolute number of oscillating genes. The first evidence for the existence of peripheral clocks, circadian oscillators outside of the SCN, came from the observation that each of the three *Per* genes oscillated in mouse liver and skeletal muscle (Zylka et al. 1998). Early evidence for the existence of a kidney clock came from rats and mice in which oscillation of *Bmal1* expression was observed in kidney tissue (Oishi et al. 1998, 2000). Confirmation of these findings came from studies in the transgenic PER2::LUCIFERASE mouse, which rhythmically expresses a PER2-LUCIFERASE fusion protein, the activity of which can be monitored by measuring bioluminescence. Tissue explants from these mice were grown in culture, revealing that peripheral tissues are capable of self-sustained circadian oscillation (Yoo et al. 2004). PER2::LUCIFERASE rhythms in the kidney oscillated ex vivo for several days in culture with a circadian period of approximately 23.5 h.

In the first study of its kind, Firsov and colleagues characterized gene expression in isolated microdissected nephron segments at discrete time points over a 24-h period in wild-type mice (Zuber et al. 2009). Specifically, global gene expression was assessed every 4 h in the DCT/connecting tubule (CNT) and the CCD. Coordinated regulation of genes in common homeostatic pathways was evident. For example, mRNA for *V2r*, encoding the vasopressin receptor important for regulating body water, reached peak expression at the same time as the mRNA for *Aqp2* and *Aqp4*, which encode aquaporin water channels. Indeed, thousands of genes were identified as oscillating transcripts in this study, firmly establishing a

critical role for the molecular circadian clock in the regulation of renal gene expression.

Mazzoccoli et al. provided convincing evidence for the molecular kidney clock with the demonstration that *Bmal1*, *Per1*, *Per2*, *Cry1*, *Cry2*, and *Reverba* exhibited significant circadian rhythms in expression in the kidney of wild-type C57BL/6 mice maintained in a 12 h:12 h light–dark cycle (Mazzoccoli et al. 2012a). Additional evidence for a kidney clock came from nonmammalian species with the finding that *Clock* oscillated in the kidney of Zebrafish (Whitmore et al. 1998, 2000). Intriguing reports focused on the fetal kidney demonstrate that clock genes and clock target genes begin oscillating during development (Landgraf et al. 2015; Meszaros et al. 2014), further supporting a role for circadian rhythms in the regulation of renal function.

6.2 Circadian Rhythms in Renal Function

6.2.1 Oscillating Aspects of Renal Function: Urine, GFR, Renal Blood Flow, and Electrolyte Excretion

The earliest recorded observation of a circadian rhythm to urine output in humans dates back to the nineteenth century [reviewed in Pons et al. (1996a) and Stow and Gumz (2011)]. Renal function oscillates in a circadian manner with daily fluctuations in renal blood flow and GFR (Vagnucci et al. 1969) and the excretion of electrolytes such as sodium and potassium (Moore-Ede 1986). Like most organs in the body, the kidney exhibits circadian oscillations in many aspects of function. In 1933, Robert Manchester reported on the diurnal patterns in urine output and renal sodium and potassium excretion. He found that these aspects of renal function were at a maximum during the day and a minimum in the night (Manchester 1933). Subsequent studies consistently showed that glomerular filtration rate (GFR) (Koopman et al. 1989), sodium excretion, and renal blood flow (Pons et al. 1996b) all vary with a circadian rhythm. Rhythms in urinary phosphate, magnesium, and acid excretion have also been observed (Cameron et al. 2007; Min et al. 1966).

6.2.2 Blood Pressure

A key function of the kidney is the maintenance of electrolyte homeostasis. A critical component of blood pressure regulation is the kidney's management of blood volume and sodium balance. Blood pressure has long been known to vary over the course of a 24-h period and this is true for rodents and humans (Agarwal 2010). Blood pressure reaches a nadir, or minimum, during the rest period and then

undergoes a sharp increase upon waking with a peak during the latter part of the active phase (Rodbard 1947; White 2000, 2008). The nadir that occurs during the rest phase is referred to as the "dip" in the circadian rhythm of blood pressure. Individuals who do not experience the nighttime 10–20 % decrease in blood pressure are termed "non-dippers." Non-dipping is very clearly associated with adverse cardiovascular outcomes, such as stroke and myocardial infarction (MI) (Chen and Yang 2015; Routledge et al. 2007). Indeed, non-dippers are known to have an increased incidence of stroke, left ventricular hypertrophy, carotid artery wall thickness and atherosclerotic plaques, microalbuminuria, cerebrovascular disease, congestive heart failure, vascular dementia, and MI (Chen and Yang 2015; Hermida et al. 2007). The mechanism underlying the connection between non-dipping and negative cardiovascular outcomes is likely related to increased organ perfusion during the rest period, leading directly to end-organ damage. Importantly, non-dipping is associated with renal damage (Chen and Yang 2015; Garcia-Ortiz et al. 2009; Leibowitz 2014).

6.2.3 Dysregulated Rhythms and Disease in the Kidney

Circadian disruption in humans is clearly associated with negative health outcomes. As just discussed, non-dipping blood pressure results in increased risk for stroke, left ventricular hypertrophy, and chronic kidney disease (CKD) (Chen and Yang 2015; Garcia-Ortiz et al. 2009; Leibowitz 2014). Below, we consider possible mechanisms related to the kidney clock and disease.

6.2.3.1 Non-dipping and the Kidney

Blood pressure regulation is multifactorial and mediated by many mechanisms including the brain and nervous system components, the vasculature, and the heart. These mechanisms of blood pressure regulation are described in more detail in Chaps. 4, 7, and 8. In this section, kidney-dependent mechanisms that may contribute to non-dipping are discussed.

Several reports have connected sodium transport in the kidney to aberrant circadian blood pressure patterns. For example, many patients suffering from hyperaldosteronism exhibit the non-dipper pattern (Uzu et al. 1998; Takakuwa et al. 2002; Williams et al. 2006) and treatment with the angiotension receptor blocker irbesartan corrected the non-dipper pattern in salt-sensitive hypertension patients (Polonia et al. 2003). Clinical studies have revealed that the diuretic hydrochlorothiazide, which inhibits NCC in the DCT, restored an appropriate decrease in nocturnal mean arterial pressure (MAP) in patients with the non-dipping phenotype, but had no effect in patients with a normal dipping pattern (Uzu and Kimura 1999). Furthermore, salt-sensitive essential hypertension is associated with non-dipping (Uzu et al. 1996) and dietary sodium restriction is able to

restore the nocturnal dipping pattern (Uzu et al. 1999). Accumulating evidence suggests that there is a direct correlation between the non-dipping pattern and a decline in renal function. For instance, non-dipping is associated with an increased risk of nephropathy (Fukuda et al. 2006) and chronic kidney disease (Portaluppi et al. 1991). Importantly, the dipping pattern can be restored after renal transplantation (Gatzka et al. 1995). Collectively, these data establish a link between abnormal circadian patterns in blood pressure and inappropriate renal sodium transport.

6.2.3.2 Chronic Kidney Disease

The incidence of chronic kidney disease (CKD) is increasing and it is recognized as a worldwide health problem (Go et al. 2014). CKD is defined as either or both reduced GFR and increased urinary protein excretion. Stage I CKD is diagnosed at an estimated GFR of less than or equal to 90 mL/min/1.73 m^2, whereas the last Stages (4–5) of CKD are associated with an estimated GFR of less than 29 mL/min/1.73 m^2. Hypertension is a key risk factor for CKD and CKD is clearly associated with adverse cardiovascular outcomes. Not surprisingly, non-dipping occurs often in CKD and reverse dipping may also occur as CKD worsens and GFR declines (Davidson et al. 2006; Portaluppi et al. 1991). (See Chap. 12 for a discussion on the use of chronotherapy in the treatment of non-dipping hypertension in CKD.)

The mechanisms underlying these effects are not well understood. In a small but important clinical study, Webb and colleagues investigated the role of Endothelin-1 (ET-1) in blood pressure dipping and arterial stiffness in CKD patients (Dhaun et al. 2014). Twenty-four hour variations in blood pressure and arterial stiffness were measured in CKD patients and healthy volunteers. Nocturnal dips in these parameters were observed in healthy controls but not in CKD patients. Comparing placebo, the calcium channel blocker nifedipine, and the ET-1 receptor blocker sitaxentan in a randomized, double-blind, three-way crossover study, these investigators found that ET-1 receptor blockade, but not calcium channel inhibition, restored the nocturnal dip in blood pressure in CKD patients. These results are particularly interesting given that ET-1 and its receptors (ETA and ETB) appear to be regulated by the circadian clock mechanism (Hwang et al. 1998; Richards et al. 2014b; Stow et al. 2012). Although additional and larger studies are needed to determine the role of circadian clock genes and ET-1 in CKD in humans, as discussed below, rodent studies have shed some light on possible circadian mechanisms underlying CKD.

An established model of CKD in rodent models is 5/6 nephrectomy in which one kidney is completely removed and the remaining kidney is ablated by surgical removal of both renal poles, leaving behind about 1/6 of the original kidney tissue. This model reproduces several hallmarks of CKD in humans, including reduced GFR and increased urine protein excretion. Hsu et al. used a rat 5/6 nephrectomy model to explore the role of circadian rhythms in the sleep disturbances that are associated with CKD (Hsu et al. 2012). Sleep quality was assessed using

electroencephalography and electromyography recordings in control versus CKD rats. Sleep disturbances were evident in CKD rats and were associated with increased mRNA expression of *Per1* and *Per2* in the hypothalamus, suggesting a role for the molecular clock in the disturbed sleep architecture associated with CKD. Sleep disturbances are commonly associated with end-stage renal disease in humans (De Santo et al. 2006; Koch et al. 2009). Interestingly, a small clinical study demonstrated that patients on conventional daytime dialysis had worse sleep than nocturnal dialysis patients and this was associated with impairment of the melatonin rhythm (Koch et al. 2010a). Melatonin amplitudes are known to correlate with GFR (Koch et al. 2010b) and kidney transplantation slightly ameliorated nocturnal sleep quality, without changes in melatonin levels or circadian rhythmicity (Russcher et al. 2015). Larger human studies are needed to increase our understanding of how the clock and kidney function are connected to sleep regulation in CKD patients.

Further support for the role of clock genes in CKD came from another rat 5/6 nephrectomy model in which expression of *Bmal1*, *Per2*, and *Dbp* (D-site albumin binding protein, a clock-controlled gene) was assessed over a 24-h period in the kidneys of control versus the remnant kidney of CKD rats (Huang et al. 2013). In this characterization study, mRNA levels were measured and protein expression was evaluated by western blot using kidney samples from animals euthanized at 4 h intervals over a 24-h period. Robust rhythms in mRNA and protein expression were observed for BMAL1, PER2, and DBP in both control and CKD rats. Cosinor analysis (analysis of biological rhythms by fitting the raw data to a cosine-based model) revealed significant differences between control and CKD rats in the mesor (circadian rhythm-adjusted mean), amplitude, and acrophase (peak of the 24 h rhythm) for all three genes tested. Additionally, protein expression and localization were evaluated using immunohistochemistry at the peak and trough time points determined by Western blot results. These results should be considered relative to the light cycle. The term "Zeitgeber ('time giver') time" (ZT) is used to denote the time with respect to the time the lights were turned on. For example, ZT0 is defined as the time the lights go on and ZT12 is the time the lights go off on a 12 h:12 h light:dark cycle. In control rats, PER2 expression was highest at ZT12 and mainly observed in proximal tubule cells at the corticomedullary junction, where the cortex and medulla meet. In contrast, PER2 expression was shifted to the distal nephron in CKD rat kidney and expression peaked at ZT8. BMAL1 expression was localized to glomerular capillaries and tubulointerstitial microvasculature in both groups, but protein expression peaked at ZT0 in control rats compared to ZT20 in CKD rats. Changes in peak/trough ratios and time of peak expression for BMAL1 and PER2 in CKD rat kidney compared to control demonstrate that disrupted clock gene expression occurs in CKD. Further mechanistic and clinical studies are needed to delineate whether altered expression of clock genes contributes to altered renal function rhythms in CKD.

6.2.3.3 Diabetes

Diabetes can be both cause and consequence of circadian clock disruption. *Clock*$^{\Delta 19}$ mice (these mice express a dominant negative form of the CLOCK protein, see Chap. 1) exhibit a diabetic phenotype characterized by reduced glucose tolerance and decreased insulin secretion (Marcheva et al. 2010). Conversely, induction of diabetes using the streptozotocin (STZ) model in rats led to the disruption of circadian clock gene expression in the kidney of diabetic rats compared to control (Soltesova et al. 2013). Specifically, *Npas2* and *Per2* expression underwent a phase advance in STZ compared to control rats. Likewise in the diabetic *db*/*db* mouse, the 24 h rhythm of *Per1*, *Reverbα*, and *Dbp* mRNA was suppressed in the kidney (Su et al. 2012). A more in-depth consideration of the circadian clock in diabetes can be found in Chaps. 2 and 5. A role for chronotherapy in the treatment of hypertension in diabetes is explored in Chap. 12.

6.2.3.4 Renal Cell Carcinoma

Renal cell carcinoma of the clear cell type (cRCC) is the most common form of malignancy in the kidney. Mazzoccoli and colleagues were the first to characterize clock gene mRNA expression in human primary cRCC tumors compared to matched normal tissue using microarray and quantitative real-time PCR (Mazzoccoli et al. 2012b). *PER2* and *TIMELESS* were downregulated in tumor tissue relative to matched normal tissue, whereas *CSNK1E* (which encodes CK1ε) was upregulated nearly twofold in tumors relative to normal tissue. Accordingly, in a subsequent study, the expression of clock genes was evaluated in renal cancer cell lines and the rhythmic expression of the *PER2* gene was measurable only in Caki-2 cells, although the protein was not detectable (Okabe et al. 2014). This may have been due to posttranscriptional regulatory mechanisms acting on *PER2* mRNA. In Caki-2 cells, the amplitude of *PER2* oscillation was augmented by direct binding of hypoxia-inducible factor (HIF)-1α to the HIF-binding site located on the *PER2* promoter. The interplay between clock genes and the hypoxic response pathway could play a key role in renal cancer, considering that this neoplasm is characterized by severely hypoxic regions. Hypoxia triggers ATR (ataxia telangiectasia and Rad3-related) activation in addition to expression of HIF-1α (Fallone et al. 2013), and tumor cells adapt to hypoxia through ATR, ATM, and DNA-dependent protein kinase (DNA-PK) involvement. Oxygen deprivation causes replicative stress establishing regions of single-stranded DNA at stalled replication forks and the activation of ATR (Fallone et al. 2013). TIMELESS protein interacts with the ATR system working in the DNA damage checkpoint response (Unsal-Kacmaz et al. 2005) and could play a role in cellular adaptation in hypoxic tumors (Bonny et al. 2013; Mazzoccoli et al. 2014).

Our understanding of how the clock mechanism may contribute to kidney cancer continues to develop. Using a xenograft model, Okazaki and colleagues

demonstrated that renal tumors exhibited circadian rhythms in expression of the key protein synthesis protein mTOR (mammalian target of rapamycin) (Okazaki et al. 2014). Optimization of the time of delivery ("chronotherapy") of mTOR inhibitor improved survival in this in vivo model of RCC. Chronotherapy in the treatment of cancer in humans is further addressed in Chap. 11.

6.3 Molecular Mechanisms of the Kidney Clock

6.3.1 Proximal Tubule and NHE3

The first molecular evidence linking the circadian clock mechanism to regulation of a key renal transport gene came from Okamura and colleagues. They showed that *Slc9a3*, the gene encoding the sodium/hydrogen exchanger NHE3, was under direct regulation by BMAL1:CLOCK heterodimers (Saifur Rohman et al. 2005). BMAL1:CLOCK was bound to an E-box element in the *Slc9a3* promoter to regulate its transcription. The circadian expression of NHE3 mRNA was blunted in *Cry1/2* knockout (KO) mice, providing further evidence that *Slc9a3* is a clock-controlled gene in the kidney. Of note, NHE3 protein expression was evaluated using immunohistochemistry in the rat kidney at two distinct time points, CT8 and CT20. As opposed to ZT, CT refers to circadian time or time in the absence of light as a time-giving cue. This usually correlates to conditions of total darkness in studies involving rodent models. NHE3 expression was localized to the brush border of the proximal tubule as expected. NHE3 protein levels were significantly higher at CT20, during the rodent active phase, compared to CT8, during the rest phase.

6.3.2 Distal Convoluted Tubule and NCC

The primary mode of entry for sodium in the distal convoluted tubule (DCT) is via the sodium chloride cotransporter (NCC). NCC is encoded by the gene *Slc12a3*. The thiazide-sensitive NCC is expressed in the apical membrane of cells in the distal convoluted tubule of the kidney where it mediates sodium and chloride entry into the cell. As discussed in Sect. 6.1.2, PER1 must be phosphorylated by CK1δ/ε in order to enter the nucleus. Thus, pharmacological inhibition of CK1δ/ε is an indirect method of inhibiting PER1. Using a model of the DCT, mDCT15 cells, the Gumz lab demonstrated that either siRNA-mediated *Per1* knockdown or treatment with the CK1δ/ε inhibitor resulted in decreased NCC expression (Richards et al. 2014a). *Per1* heterozygous mice exhibited reduced levels of NCC mRNA in the renal cortex compared to wild-type control mice. Consistent with this, CK1δ/ε inhibition in wild-type mice resulted in reduced PER1 levels in the nuclear fraction

from the renal cortex. Further supporting a role for PER1 and CK1δ/ε in the regulation of NCC, NCC mRNA expression was reduced in CK1δ/ε inhibitor-treated wild-type mice compared to vehicle-treated controls. Importantly, CK1δ/ε inhibitor treatment resulted in decreased NCC activity in mDCT15 cells, suggesting that regulation of NCC by PER1 and CK1δ/ε extends from the transcriptional level to the level of transporter activity.

An important mechanism of NCC regulation involves a complex kinase pathway that is still being characterized. The active form of NCC at the apical membrane of the DCT is phosphorylated by the serine–threonine kinases: oxidative stress responsive kinase 1 (OSR1) and Ste20-related proline–alanine-rich kinase (SPAK). OSR1 and SPAK are also subject to phosphoregulation through a cascade involving members of the with-no-Lysine kinase (WNK) family. Our investigation into Per1-mediated regulation of NCC, WNK1, WNK4, and a kidney-specific splice variant of WNK1, designated KS-WNK1, revealed that Per1 coordinately regulates members of this pathway in a manner that is consistent with a positive role for Per1 in the regulation of NCC (Richards et al. 2014a). For example, siRNA-mediated knockdown of *Per1* and CK1δ/ε inhibition separately resulted in the upregulation of WNK1 mRNA but downregulation of the NCC-inhibitory kinases WNK4 and KS-WNK1. Although this investigation was limited to a transcription-based mechanism of regulation, these findings were consistent with the work of Susa et al. These investigators demonstrated that levels of phospho-OSR, phospho-SPAK, and phospho-NCC varied with a circadian pattern (Susa et al. 2012). Interestingly, these investigators found that the rhythmic variation in phospho-NCC was ablated by the administration of the aldosterone antagonist eplerenone, suggesting a role for aldosterone in circadian clock-mediated regulation of the NCC pathway. Aldosterone synthesis is rhythmic in humans (Liddle 1966) and rodents (DeForrest et al. 1979) with a peak in the early part of the active phase. Connections between aldosterone signaling and the molecular clock in the kidney will be discussed further below.

Aldosterone and vasopressin modulate ion transporters and channels in the kidney that are crucial for sodium and water balance such as ENaC and the thiazide-sensitive NCC (Eladari et al. 2014; Pearce et al. 2015). In a small clinical study, Castagna et al. evaluated in urinary exosomes the diurnal pattern of expression of NCC and prostasin (Castagna et al. 2015). Prostasin is an extracellular serine protease with trypsin-like activity involved in the proteolytic activation of ENaC and encoded by *PRSS8* gene, and it is expressed with circadian rhythmicity in the mouse kidney (http://bioinf.itmat.upenn.edu/circa/, last accessed 5-16-2015). In urinary exosomes of healthy subjects, levels of NCC and prostasin showed circadian variation with an acrophase in the late afternoon, in line with ADH (antidiuretic hormone) and AQP2, consistent with a role for the molecular kidney clock and both prostasin and NCC in maintaining water balance.

6.3.3 Collecting Duct and ENaC

Our studies on PER1 in the kidney began with the finding that aldosterone acutely induced *Per1* mRNA expression in murine renal inner medullary collecting duct cells (mIMCD-3) (Gumz et al. 2003). We subsequently hypothesized that PER1 may mediate downstream effects of aldosterone on renal sodium transport genes. This hypothesis led to the finding that siRNA-mediated knockdown of PER1 in several immortalized murine renal collecting duct cell lines (mpkCCD$_{c14}$, OMCD-1, mIMCD-3, and IMCD-K2) resulted in decreased expression of *Scnn1a*, which encodes the alpha subunit of ENaC (Gumz et al. 2009, 2010). In vivo evidence further supported a role for PER1 in the regulation of ENaC; αENaC mRNA levels were reduced in the renal cortex, outer medulla, and inner medulla of *Per1* KO mice compared to WT (Gumz et al. 2009, 2010). We next found that pharmacological inhibition or siRNA-mediated knockdown of CK1δ/ε recapitulated the effects of Per1 knockdown in mpkCCD$_{c14}$ cells, resulting in decreased mRNA and protein expression of αENaC (Richards et al. 2012). Using two different in vitro models of renal collecting duct cells, amphibian A6 cells and murine mpkCCD$_{c14}$ cells, we demonstrated that CK1δ/ε inhibition resulted in decreased ENaC activity. Together these results established a role for PER1 in the regulation of αENaC subunit expression and ENaC activity in renal collecting duct cells.

6.4 Regulation of the Kidney Clock

6.4.1 Light and Food

Light is a key zeitgeber in entrainment of the central clock in the SCN (see Chap. 1), but food is an important synchronizing cue as well. Peripheral clocks are sensitive to changes in the timing of nutrient cues than to changes in light, for example. In a convincing study, Schibler and colleagues demonstrated that time-restricted feeding uncoupled circadian oscillation in peripheral tissues from that in the SCN (Damiola et al. 2000). Animals placed in constant darkness were restricted to feeding during the rest phase, resulting in peripheral tissues oscillating out of phase with SCN oscillation. Wu et al. investigated this by looking at the effects of only reversing the light/dark (LD) cycle, restricting feeding to the daytime or reversing both the LD cycle and feeding time on clock gene expression in rat kidneys (Wu et al. 2010). LD reversal did not alter the expression pattern of *Bmal1*, *Cry1*, *Clock*, or *Per2* but did delay the peak expression of *Per1* by 4 h. In addition, the expression levels of *Per1*, *Cry1*, *Clock*, and *Bmal1* were altered only with the reversal of the LD cycle. Feeding time reversal caused peak expression shifts of 8–12 h for *Clock*, *Cry1*, and *Bmal1* after 7 days while causing 4 h shifts in *Per1* and *Per2*. Combining LD and feeding time reversal resulted in a total reversal of the circadian expression of all five genes studied within 7 days. This evidence

points to the cumulative effects of light and food cues regarding the entrainment of peripheral clocks.

The work of Wu and colleagues clearly demonstrates that the timing of nutrient delivery affects circadian clock gene expression in the kidney. Oishi et al. found that the type of dietary nutrients also affects clock gene expression in the kidney (Oishi et al. 2012). Mice were fed a low-carbohydrate, high-protein (HPD) diet for 2 weeks. The central clock did not appear to be altered since locomotor activity and body temperature rhythms were not different between HPD and normal diet groups. Phase advances in gene expression were observed for *Bmal1*, *Cry1*, *Npas2*, and *Rev-erbα*, but not *Per2*, in the kidney (and liver) of the HPD-fed mice compared to normal diet mice, demonstrating again the role of food cues in the entrainment of peripheral clocks.

Mochel et al. also demonstrated a role for food cues in modulating circadian rhythmicity of urinary sodium and potassium and blood pressure in dogs. Feeding time impacted the circadian pattern of renin activity (see Sect. 6.4.3 for more details on the renin signaling cascade in the kidney), urinary electrolyte excretion, and blood pressure regulation, seemingly through food-related signals, for instance, dietary sodium, which synchronize circadian oscillators downstream of the central oscillators ticking in the suprachiasmatic nucleus (Mochel et al. 2014).

6.4.2 Temperature

Internal body temperature and external environmental temperature are known to act as a synchronizing input to the clock; neurons in the SCN can be entrained by temperature (Brown et al. 2002). The mechanism of these effects is still being elucidated, but convincing evidence suggests a role for RNA-binding proteins such as CIRP (cold-inducible RNA-binding protein) which has been shown to post-transcriptionally regulate *Clock* (Morf et al. 2012) and HSF1 (heat shock factor 1) which activates *Per2* expression (Chappuis et al. 2013). For an excellent review on this topic, see Ki et al. (2015).

Little is known about the role of temperature in the regulation of the kidney clock. In a very interesting study using transgenic PER2::LUCIFERASE mice, Ohnishi and colleagues tested the effect of water bath temperature on circadian phase-shifting (Ohnishi et al. 2014). In vivo bioluminescence recordings were made in live animals. Raising core body temperature to 41 °C resulted in phase advances of circadian PER2::LUCIFERASE activity in the kidney, liver, and submandibular gland. Future studies aimed at determining the role of temperature in circadian regulation of renal function are needed to increase our understanding of how this synchronizing cue affects the kidney clock.

6.4.3 Hormonal Regulation of the Kidney Clock

The kidney is both a target and generator of hormonal regulation. The renin angiotensin aldosterone system (RAAS) is a critical regulator of volume and sodium balance. Renin is produced in the kidney in the cells of the juxtaglomerular apparatus (JGA) in response to the decrease in plasma sodium, blood volume, or blood pressure. Renin converts angiotensinogen to angiotensin I which is then converted to angiotensin II (AngII) by the angiotensin-converting enzyme (ACE). AngII is a potent vasoconstrictor and acts to increase blood pressure through myriad actions, including induction of aldosterone production by the adrenal gland. AngII and aldosterone both act on tubular cells in the kidney to upregulate sodium reabsorptive mechanisms. For example, ENaC activity in the CCD is increased in response to AngII and aldosterone (Mamenko et al. 2012). Plasma renin, AngII, and aldosterone levels all vary with a circadian rhythm in humans (Kawasaki et al. 1990). Interestingly, in a small clinical study, angiotensinogen levels did not appear to change with time of day in healthy subjects when levels were evaluated in urine (Isobe et al. 2015). In CKD patients, however, higher levels of angiotensinogen were evident in the daytime and this time-dependent variation was paralleled by circadian fluctuation of albuminuria and proteinuria. The circadian pattern was absent in patients with reversed dipping blood pressure oscillation, suggesting a role for altered circadian rhythmicity of the intrarenal RAAS in determining hypertension and renal damage.

These observations demonstrate multilayered regulation of renal function by the clock: circadian clock proteins act in discrete kidney cell types to directly regulate expression of genes critical to renal function such as sodium transporters, while at the same time, clock proteins act in separate tissues such as the adrenal gland (see Sect. 6.5.2) to regulate the synthesis of hormones that act on the kidney to regulate its function. As discussed in the next section, loss of circadian protein expression results in dramatic changes in renal function and blood pressure.

6.5 Renal and Blood Pressure Phenotypes in Rodent Circadian Mutant Models

6.5.1 CLOCK Regulates Blood Pressure and Renal Function

Clock KO mice are hypotensive, meaning they have low blood pressure. The average mean arterial pressure and mean systolic blood pressure were significantly lower in *Clock* KO mice compared to WT (Zuber et al. 2009). Although these mice maintained a normal 24 h rhythm of BP, they displayed altered urinary Na excretion rhythms and a mild diabetes insipidus. *Clock* KO mice also have altered circadian expression patterns of 20-HETE (20-hydroxyeicosatetraenoic acid) (Nikolaeva et al. 2012). 20-HETE can lead to either increases or decreases in blood pressure

through actions on the kidney. If acting on preglomerular arterioles, the vasoconstrictive action of 20-HETE raises blood pressure. However, it is also capable of blocking channels and transporters which reabsorb sodium in the thick ascending limb and proximal tubule of the nephron which leads to a decrease in blood pressure (Williams et al. 2010). Therefore, Firsov et al. have proposed the dysregulation of 20-HETE to be a contributing factor to the blood pressure phenotype of *Clock* KO mice.

6.5.2 Salt-Sensitive Hypertension in CRY1/CRY2 Knockout Mice

Doi et al. characterized the salt sensitivity of blood pressure in *Cry1*/*Cry2* KO mice (Doi et al. 2010). These mice had normal blood pressure when maintained on a normal diet; however, they exhibited a salt-sensitive hypertension in response to a high sodium diet. The high sodium-induced increase in blood pressure was ameliorated in *Cry1*/*Cry2* KO mice by treating with eplerenone, an aldosterone antagonist that blocks the mineralocorticoid receptor to which aldosterone binds. The normal circadian rhythm of plasma aldosterone levels was disrupted in *Cry1*/*Cry2* KO mice. As well, total plasma aldosterone was dramatically higher in *Cry1*/*Cry2* KO mice compared to wild-type control mice. To uncover the mechanism of this effect, these investigators performed a microarray analysis on aldosterone-producing cells of the zona glomerulosa of the adrenal gland, revealing significant overexpression of *Hsd3b6*. *Hsd3b6* encodes 3β-hydroxyl-steroid dehydrogenase (3βHSD), a dehydrogenase isomerase in the aldosterone synthesis pathway. Increased activity of this enzyme was recorded in the adrenal gland of *Cry1*/*Cry2* KO mice and linked to the observed salt-sensitive hypertension.

6.5.3 Regulation of Blood Pressure and Renal Sodium Handling by Per1

As described in Sect. 6.3.3, our initial studies of PER1 in the kidney began with the observation that *Per1* is an aldosterone target gene (Gumz et al. 2003). To follow up the finding that PER1 regulates the aldosterone target αENaC in collecting duct cells, blood pressure was examined in global *Per1* KO mice. On a normal diet and on a normal light:dark cycle, *Per1* KO mice exhibited mean arterial blood pressure that was 18 mm Hg lower than wild-type control mice (Stow et al. 2012). This reduced blood pressure phenotype was observed on the 129/sv background strain. The 129/sv strain of mice exhibits baseline blood pressure that is higher than that of the C57BL/6 mouse strain (Hartner et al. 2003). Thus, loss of PER1 appears to be protective for blood pressure in a setting of spontaneous hypertension.

The observation that PER1 regulates the critical sodium channel ENaC suggested that PER1-mediated regulation of blood pressure could be related to sodium handling in the kidney. In order to characterize the role of PER1 in renal sodium handling, mice with reduced PER1 expression were subjected to a reduced sodium diet. Surprisingly, these *Per1* heterozygous mice appeared to excrete more sodium in the urine than wild-type control mice even under normal dietary conditions (Richards et al. 2013). *Per1* heterozygous mice responded appropriately to a low sodium diet by dramatically reducing urinary sodium excretion, but these values remained higher than wild-type control mice. Examination of plasma aldosterone levels at two distinct time points (ZT6 and ZT18, noon and midnight) in the circadian cycle demonstrated that *Per1* heterozygous mice failed to increase plasma aldosterone during the active phase. Moreover, plasma aldosterone levels were much lower in *Per1* heterozygous mice at both ZT6 and ZT18. To determine the underlying cause of the reduced plasma aldosterone levels, gene expression was examined in the adrenal glands of wild type compared to *Per1* heterozygous mice at ZT6 and ZT18. Expression of *Cyp11b2*, the gene encoding the aldosterone synthase enzyme, was not different between wild-type and *Per1* heterozygous mice. *Hsd3b6* expression was much lower in *Per1* heterozygous mice, however, and the time-dependent increase in expression of this gene observed in wild-type mice was absent in *Per1* heterozygous mice. These results suggested a role for PER1 in the positive regulation of *Hsd3b6* and aldosterone production. Interestingly, this phenotype was opposite to that of *Cry1*/*Cry2* double KO mice, discussed above, which exhibited increased levels of *Hsd3b6* in the adrenal gland along with dramatically higher plasma aldosterone levels (Doi et al. 2010). These apparently opposing actions of PER1 and CRY1/CRY2 are surprising given that these circadian clock proteins were both characterized according to their negative regulatory actions in the transcription translation feedback loop of the clock mechanism. As discussed in Chap. 1, these findings are an example of circadian clock proteins behaving in a surprising way that is not easily explained. Future studies using tissue-specific and cell type-specific KO of *Per1* and *Cry1*/*Cry2* may shed light on the mechanism underlying these conflicting actions.

6.5.4 Cardiorenal Phenotype of Tau Mutant

The *tau* mutant golden hamster is an intriguing model for the study of circadian disruption. These rodents contain a gain-of-function mutation in CK1ε that results in a shorter circadian period or tau of 20 h compared to 24 h (Lowrey et al. 2000; Ralph and Menaker 1988). Tau heterozygotes (+/*tau*) have decreased life expectancy and exhibit a tau of 22 h. Martino et al. investigated possible mechanisms for this phenotype and discovered a profound cardiorenal phenotype in +/*tau* hamsters (Martino et al. 2008). These rodents exhibited dramatic cardiac and renal fibrosis and hypertrophy that correlated with early mortality. Moreover, +/*tau* hamsters displayed proteinuria, tubular cell apoptosis, and tubular dilation. When the +/*tau*

hamsters were maintained on their endogenous 22 h light:dark cycle compared to a 24 h cycle, starting at the age of 4 months (before the cardiorenal phenotype is evident), the cardiorenal phenotype did not appear and longevity was restored. In a separate maneuver, these investigators attempted to reverse the +/*tau* phenotype via SCN ablation at the age of 6 months. This resulted in the correction of cardiac hypertrophy in +/*tau* hamsters, but the renal phenotype was not examined. This outcome presents a very interesting phenomenon—desynchrony between the central and peripheral clocks. That is, the SCN in the +/*tau* hamster operates on the 24 h cycle driven by the normal light:dark cycle in the animal facility, but the peripheral clocks are conflicted between operating on the animal's endogenous 22 h cycle and the 24 h signals received from the SCN clock. This desynchrony was clearly associated with a severe cardiorenal phenotype and early death. Placing the animals on their inherent 22-h cycle prevented development of the cardiorenal phenotype and corrected mortality as well. As Dr. Frederick Turek so eloquently wrote in his commentary on Martino et al.'s report, "no rhythm is better than bad rhythm" (Turek 2008).

6.5.5 Blood Pressure Regulation by Other Circadian Proteins

6.5.5.1 BMAL1 Regulates Blood Pressure

In 2007, Curtis et al. described the blood pressure phenotype of the *Bmal1* KO mouse (Curtis et al. 2007). While the wild-type mice exhibited normal circadian variations in heart rate and blood pressure, *Bmal1* KO exhibited a non-dipper phenotype with a 24 h blood pressure that was lower than that of the wild-type control mice. Plasma norepinephrine and epinephrine were dramatically reduced in *Bmal1* KO mice compared to wild-type. These findings clearly demonstrated a role for BMAL1 in blood pressure regulation and suggested that sympathoadrenal signaling may contribute to the mechanism of that regulation.

6.5.5.2 Cardiovascular Phenotype in *Per2* Mutant Mice

Vukolic et al. characterized the cardiovascular phenotype of the *Per2* mutant mouse (Vukolic et al. 2010). This mouse expresses a mutated form of PER2 that lacks the C-terminal region of the PAS B domain and the entire PAC domain. These mutant PER2 proteins are truncated relative to the wild-type protein, lacking possible dimerization sites. *Per2* mutant mice displayed a higher heart rate during the light period than wild-type mice, but locomotor activity rhythms were similar. On a standard 12 h light:dark cycle, *Per2* mutant mice exhibited decreased 24 h diastolic blood pressure, increased heart rate, and decreased difference between day and night blood pressure.

6.5.5.3 Ancillary Circadian Transcription Factors Regulate Blood Pressure

Wang et al. characterized the blood pressure phenotype of mice lacking three ancillary circadian transcription factors, DBP (D-site binding protein), TEF (thyrotroph embryonic factor), and HLF (hepatic leukemia factor) (*Dbp/Tef/Hlf* triple KO) (Wang et al. 2010). While single or double KOs of these factors do not produce much of a phenotype, triple KO mice usually do not live past 1 year. This may be due to the fact that the three factors have very conserved amino acid sequences and are able to compensate for the loss of one another. Similar to the loss of the core clock proteins already discussed, the triple KO of *Dbp/Tef/Hlf* resulted in a blood pressure phenotype. The triple KO mice exhibited the normal circadian rhythm of blood pressure, but their mean arterial pressure was significantly lower (~25 mm Hg) than that of wild-type control mice. This phenotype was associated with cardiac hypertrophy, reduced plasma aldosterone, and increased urine sodium excretion. Although renal ENaC activity was not measured, it is interesting to note that colonic ENaC activity (measured by potential difference in the distal colon) was significantly reduced in triple KO mice and the circadian rhythm of distal colon ENaC observed in wild-type control mice was absent in the triple KO mice.

6.5.6 The First Kidney-Specific Knockout of a Circadian Rhythm Gene

The first kidney-specific KO of a clock gene was generated using floxed *Bmal1* mice crossed with Renin-Cre (Tokonami et al. 2014). The resulting mice lacked BMAL1 in renin-producing cells in the kidney, which are localized to the juxtaglomerular apparatus. However, reduced BMAL1 expression was also observed in the liver and in other regions of the kidney. Notably, BMAL1 expression was significantly decreased in the mTAL, CCD, and OMCD. Mice with kidney-specific KO of *Bmal1* exhibited decreased plasma aldosterone compared to control mice. Loss of BMAL1 in specific renal cell types in these animals was also associated with significantly lower blood pressure compared to control mice and the 24 h rhythm of plasma aldosterone was disrupted as well. Significant reduction in plasma and urine osmolality was observed as well. Importantly, GFR was increased in kidney-specific *Bmal1* KO and the time-dependent increase in GFR at midnight relative to noon was not affected. Importantly, blood pressure was significantly decreased in the kidney-specific *Bmal1* KO mice compared to control mice.

6.6 Conclusions

Clinical observations regarding oscillations in renal function have been established for decades, if not longer. Advances in understanding the molecular clock in the recent past provide a putative mechanism underlying the circadian variations in renal function. Regulation of renal sodium transporters by circadian clock proteins, for example, may contribute to circadian variations in urinary sodium excretion. Circadian regulation of sodium and water homeostasis by the kidney clock likely contributes to blood pressure rhythms. Much work remains to be done. Given the multitude of specialized cell types in the kidney, specific KO of clock proteins in discrete nephron segments and cell types will greatly increase our understanding of the mechanisms by which the circadian clock contributes to regulation of renal function.

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Chapter 7
Circadian Rhythms and the Circadian Clock in the Cardiovascular System

R. Daniel Rudic

Abstract Circadian rhythms play an important role in the cardiovascular system, affecting normal physiology and pathology. While circadian rhythms in blood pressure have long been known, the emergence of our understanding of the tissue pervasiveness and ubiquity of circadian clocks has elucidated additional roles of clocks in the cardiovascular system with regard to physiology, signaling, and disease. The key conduit of the circulatory system, the blood vessels, has their own vascular clocks as do all crucial cardiovascular homeostasis controlling organs such as kidney and heart. The circadian clock has emerged to have an intimate relationship with regulators of vascular function, interacting with both paracrine and autocrine signals. Angiotensin II, eNOS, Akt, and other key regulators of cardiovascular function are affected by clocks. While much of our understanding has occurred through the use of animal models with mutations of clocks, clock dysfunction does occur in humans and is relevant with regard to sleep disorders, shift work, and even aging. Our bodies are gauged to have circadian timing truly critical to health and may offer new insights into improved approaches to therapeutics and disease.

Keywords Circadian • Vascular • Endothelium • eNOS • Angiotensin • Bmal1 • Clock • Vascular remodeling • Blood pressure • Hypertension • Diabetes • Obesity

7.1 Introduction

The circadian clock is a signaling pathway that comprises the molecular basis of circadian rhythms (see Chap. 1 for a detailed introduction to the molecular mechanism of the clock). In the nervous system, the circadian clock is responsible for generating rhythmic behavior. The cardiovascular system of mammals also follows a circadian rhythm, exhibiting a genetic and functional oscillation of approximately 24 h. Physiologically, blood pressure follows a 24-h profile, rising in the daytime

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and falling at night. Heart rate, endothelial function, circulating levels of humoral signals, and myogenic tone also follow a circadian rhythm. Indeed, the cardiovascular system is fully equipped with the same components of the circadian clock that are found in the SCN of the brain. The organs that control the function of the circulatory system [blood vessels, kidney (see Chap. 6), heart (see Chap. 8)] have an oscillating circadian clock. Each of these organs, composed of a heterogeneous composition of cell types including endothelial cells, smooth muscle cells, epithelial cells, cardiac myocytes, and fibroblasts, has its own circadian clock. Herein, the importance of the circadian clock and circadian rhythms will be discussed with regard to their significance in cardiovascular cell signaling, blood pressure control, vascular function, and disease based on insights from human physiology and experimental animal models.

7.2 Oscillating Clocks in Blood Vessels

The diurnal variation in sleep and locomotor activity emanates from rhythmic oscillation of clock genes in the SCN of the brain. Oscillating circadian clocks are also present in the vasculature. BMAL1, CLOCK, NPAS2, PER, and CRY proteins, which comprise the core circadian clock, are expressed and oscillate in vascular tissue (Rudic et al. 2005b; McNamara et al. 2001). The vascular tree is complex, composed of arteries, veins, and microvessels which are different in their composition with regard to smooth muscle cells and lamina. However, one commonality across the vascular tree is the expression of the circadian clock. The vascular tree is functionally structured, with arteries being more elastic to propagate movement of blood, arterioles being abundant in number and propensity for contractility to control tissue delivery, and veins being pliable to amass fluid return. While all of the vasculature does express a clock, functionality and timing of the clock within the different blood vessel types is uniquely coordinated. Indeed, the timing of circadian oscillation in arteries is different than veins (as indexed by *Per1*-luciferase oscillation), and these rhythms do persist ex vivo for as many as 12 circadian cycles (Davidson et al. 2005). The differences of circadian timing in blood vessels may be of an organic origin, or alternatively, due to variations in blood flow and pressure across the vasculature. For example, the mechanical forces exerted by the pulsatile motion of blood vary in relation to proximity to the heart, and as such these forces may act as an entraining signal to the circadian clock, in a manner analogous to the light–dark cycle which resets the central clock (Czeisler et al. 1981) and food which entrains the peripheral liver clock (Damiola et al. 2000; Stokkan et al. 2001).

Circadian clocks also exhibit rhythm within vascular cells in culture. This observation stems from the ability to recapitulate circadian rhythm in cultured cells. Individual cells in vitro have been demonstrated to have an oscillating circadian clock; however, cell populations en masse are arrhythmic due to asynchronous circadian clock oscillation among cells (Nagoshi et al. 2004). Only until a

phase-synchronizing stimulus is applied to cultured cells do they elicit a uniform circadian rhythm (Welsh et al. 2004). In cultured fibroblasts, this is accomplished by a short duration, high concentration of horse serum (Balsalobre et al. 1998) or even glucose (Hirota et al. 2002) which phase aligns the circadian clock among cells, the result being a uniform oscillatory clock signal (i.e., *Bmal1*, *Per2* oscillation). The circadian clock can be modulated by vascular signaling molecules which serve to change the timing of the clock by either phase advancing or delaying the rhythm. Work in vascular smooth muscle cells has permitted the elucidation of these "vascular clock"-modifying signals. Molecular signals with established roles in the control of vascular contractility, tone, and remodeling, which include endothelin, prostanoids, angiotensin, and even nitric oxide, also impart a significant influence on the circadian clock.

7.3 Endothelial Function Is Controlled by the Circadian Clock

Blood vessels are composed of three layers, an inner endothelial cell layer that is in contact with circulating blood, an underlying layer composed of smooth muscle cells, and an outer adventitial layer composed of fibroblasts (Fig. 7.1). Each layer confers a unique functionality. The adventitia is an outer layer of connective tissue that provides a protective covering to the blood vessel. Smooth muscle cells of the tunica media comprise the middle layer of contractile cells that allow the blood

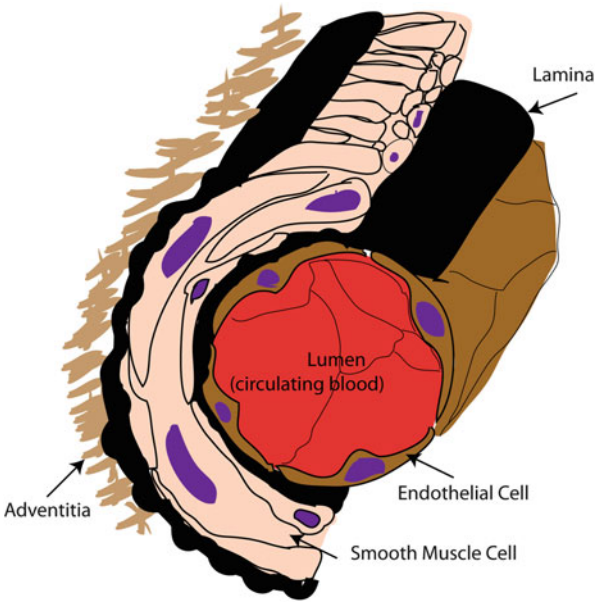

Fig. 7.1 The anatomy of the blood vessel. A blood vessel is comprised of three layers. An outer adventitial layer, a smooth muscle cell layer called the media, and an inner layer comprised of endothelial cells called the inima. Each layer is separated by a lamina (*black line*)

vessel to constrict and relax. The endothelial cells of the endothelium being in direct contact with flowing blood communicate mechanical forces caused by circulating blood into autocrine and paracrine biochemical signals to the smooth muscle and even adventitia. Indeed, with regard to the endothelium, the circadian clock plays an important role in the regulation of its function. The relaxant response to acetylcholine in aortae from *Per2* mutant mice (Viswambharan et al. 2007), *Bmal1*-KO, and $Clock^{\Delta 19}$ mutant mice (Anea et al. 2009) has been shown to be reduced. Acetylcholine relaxes blood vessels by activating endothelial muscarinic receptors to release intracellular calcium to activate the enzyme endothelial nitric oxide synthase (eNOS) to produce nitric oxide (NO) which in turn diffuses from the endothelial cell to smooth muscle cell to cause relaxation. Despite this impairment in endothelial function, vascular smooth muscle cell function is normal in these mice. There is further evidence to suggest that these impairments are directly dependent on deterioration of circadian rhythms. $Clock^{\Delta 19}$ mice have normal endothelial function in LD conditions, but develop dysfunction only when acclimated to free-running conditions (DD), conditions that are known to impair circadian rhythm in this particular genetic mutant model.

7.4 Clock Control in Long-Term Vascular Process: Remodeling and Disease

Normal endothelial function counterbalances the progression to vascular disease through a process of remodeling. As such, the endothelial dysfunction observed in circadian clock-deficient mice causes pathological responses in response to vascular injury. Blood clot formation through the process of thrombosis is accelerated during endothelial function. The process of thrombosis exhibits a circadian rhythm in WT mice as demonstrated in an experimental model of photochemically induced thrombosis, but this rhythm is absent in $Clock^{\Delta 19}$ mice (Westgate et al. 2008). While $Clock^{\Delta 19}$ mice exhibit a delay in the duration of time taken to reach blood flow arrest after the laser injury, other circadian clock mutant mice exhibit a different phenotype. Mice with endothelial specific-disruption of Bmal1 also lose rhythm in this time of day-dependent thrombosis, but in contrast suffer blood flow arrest much faster due to thrombosis, a deleterious phenotype. Thus, the circadian clock conditions acute thrombotic events.

In addition to controlling the acute endothelial and circulating blood interactions that contribute to thrombosis, the genetic components of the circadian clock also exert a significant influence in the chronic adaptation of the vascular wall and its restructuring in the process called vascular remodeling. Experimentally, vascular remodeling can be induced by surgically altering blood flow via arterial ligation (Fig. 7.2a), which in normal animals causes the blood vessel to narrow and the blood vessel wall to thicken over a time span of several weeks. In mice with a dysfunctional circadian clock, arterial ligation of the common carotid artery causes

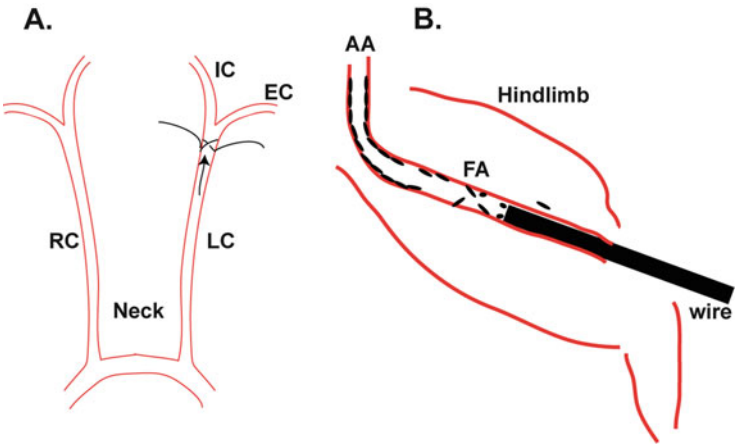

Fig. 7.2 Experimental models of vascular injury. (**a**) Ligation of the left common carotid artery (LC) by placement of a suture interrupts blood flow to the internal common carotid artery (IC) and external common carotid artery (EC). After 2 weeks of ligation, the LC narrows due to a process of vascular remodeling and is reduced in size relative to the contralateral right common carotid artery (RC). Some animals that are mutated genes exhibit an abnormal response, and instead of narrowing, exhibit a growth response, such as the circadian clock mutant mice. This model mimics the arterial blockages that can occur in human disease. (**b**) In the hindlimb or leg of mice, the femoral artery (FA) which branches from the abdominal aorta (AA) can be surgically exposed and a wire is placed inside the vessel to disrupt the endothelial layer to cause injury. Again, circadian clock mutant mice exhibit a worsened response to the wire injury than compared to normal mice. This model recapitulates the angioplasty approach that is used in medicine to treat arterial blockages

a pathological response characterized by enhanced wall thickening and a growth of the inner lining surrounding the endothelium of the blood vessel in a process called intimal hyperplasia (Anea et al. 2009). Arterial remodeling can also be assessed by direct injury to the endothelium by surgically placing a small wire inside the lumen of the blood vessel (Fig. 7.2b). Again, in circadian clock mutant mice, this wire injury causes an exacerbated injury response, relative to wild-type mice (Anea et al. 2009). Further, that these impairments occur only when $Clock^{\Delta 19}$ mice are placed in free-running conditions (DD but not LD) is further evidence that these effects are due to a circadian function of the clock genes. While large blood vessels are important in disease process such as hypertension, small blood vessel development is important in disorders such as cancer and inflammation in the process of angiogenesis. Small blood vessel development is impaired in circadian clock mutant mice. Small blood vessels sprout new vessels during conditions of metabolic stress. Ischemia in the hindlimb induced by ligation causes such angiogenesis, but the angiogenic response is reduced and results in susceptibility to limb loss in Per2 mutant mice (Gao et al. 2008). Transplant arteriosclerosis of large blood vessels is also worsened in circadian clock mutant mice. During organ transplants, blood vessels are connected to maintain blood circulation to the donor tissue. In an experimental model of blood vessel transplantation, blood vessels from circadian

clock mutant mice (*Bmal1*-knockout (KO) or *Per* isoform KO mice) suffered increased vessel disease relative to normal wild-type donor blood vessels (Cheng et al. 2011). These observations point to significant role for the circadian clock in the progression of large artery and small vessel disease, which often occurs in humans simply as a consequence of aging. Indeed, one hallmark feature of an aging vasculature is the stiffening or hardening of the blood vessels. This process of stiffening is accelerated in circadian $Clock^{\Delta 19}$ mice (Anea et al. 2010) as is the development of atherosclerosis (Pan et al. 2013).

7.5 Clocks Worsen with Aging

While there are polymorphisms of the circadian clock, the native circadian clock also worsens with aging with regard to expression and oscillation. *Bmal1* and *Per2* oscillation and expression are blunted in high passage (aged in vitro) human aortic smooth muscle cells and in vivo in large arteries (aortae) from old WT mice (Kunieda et al. 2006). Furthermore, endothelial cells and aortic tissue of *Per2* mutant mice have elevated markers of senescence (Gao et al. 2008). It has been shown that the aging process impairs clock function with consequences on the health of blood vessels. *Bmal1*-KO mice exhibit a phenotype of premature aging manifesting in hindlimb arthropathy (Bunger et al. 2005), increased end organ disease (Kondratov et al. 2006), and increased mortality (Sun et al. 2006). With regard to blood vessel disease, its progression is part of the aging process. In fact, blood vessels grow old by stiffening, losing their elasticity, hence becoming less effective in delivering blood to target organs. Interestingly, arterial stiffening is also affected by the circadian clock (Anea et al. 2010).

7.6 The Circadian Clock and eNOS

Endothelial nitric oxide synthase (eNOS) is a key enzyme in the biology of blood vessels. Blood vessels are composed of three layers, an inner endothelial cell layer that is in contact with circulating blood, an underlying layer composed of smooth muscle cells, and an outer adventitial layer composed of fibroblasts. The endothelial cells that comprise the endothelium contain eNOS, which releases nitric oxide to relax blood vessels, in an enzymatic reaction that is dependent on cofactors to bind eNOS including tetrahydrobiopterin (BH4). Increasing evidence has demonstrated that the circadian clock regulates endothelial nitric oxide synthase (eNOS) signaling. The phosphorylated form of eNOS (Kunieda et al. 2008) (P-eNOS) and its activating kinase AKT exhibit circadian oscillation in the vasculature of wild-type mice, an important pathway that enhances NO production (Dimmeler et al. 1999; Fulton et al. 1999), protects cells from apoptosis (Datta et al. 1997), and modulates vascular function (Luo et al. 2000). *Bmal1*-KO mice exhibit decreased P-eNOS

expression and AKT, which phosphorylates eNOS, is also decreased. PDK-1, which phosphorylates AKT, is also misregulated in both *Bmal1*-KO (Anea et al. 2009) and *Per2* mutant mice (Gao et al. 2008).

The relationship between nitric oxide and the circadian clock may be reciprocal. NO donors activate Per1 transcriptional activity and conversely increase Bmal1 expression in endothelial cells. eNOS-KO mice also exhibit a phase shift of the circadian clock (*Per*), while the NOS inhibitor L-NAME phase advances *Per2* expression in smooth muscle cells. That L-NAME phase shifts *Per-2* in smooth muscle cells suggests that the effect is eNOS independent, since eNOS is discretely localized to endothelial cells, but suggests that the neuronal NOS isoform (nNOS) is modulating the circadian clock in smooth muscle cells (Kunieda et al. 2008). In contrast to the effect of NO in the vasculature, neither eNOS nor nNOS-KO mice have any aberration in behavioral circadian function (Kriegsfeld et al. 1999, 2001), suggesting that endogenously produced NO may exert a vascular-specific role in control of the circadian clock. The phosphorylated, activated forms of AKT and eNOS are blunted in arteries in mice deficient in the circadian clock, a pathway known to facilitate vasodilatory responses in blood vessels (Luo et al. 2000). Aging also plays a part in these signaling mechanisms. Phosphorylated eNOS is attenuated in aged mice, while *Per2* oscillation is attenuated in aortae and vascular cells of aged wild-type mice and eNOS-KO mice, suggesting that there is an age-conditioned relationship between NO and the circadian clock that is reciprocal (Kunieda et al. 2008). Additional evidence suggests that elevation in pressor mechanisms, and in particular those mediated by COX-1, may also play a part. Cyclooxygenase-1 (COX-1) protein expression is increased in aortae of *Per2* mutant mice, and the contractile response to indomethacin is exacerbated.

7.7 The Circadian Clock Interaction with Vascular Signals

Understanding the signals that regulate the circadian clock in vascular cells may ultimately provide novel therapeutic tools to treat circadian dysfunction in the vasculature and the periphery. In culture, single cells have an oscillating circadian clock, but a population of cells is arrhythmic due to asynchronous oscillation among cells (Nagoshi et al. 2004). Thus, a phase-aligning stimulus must be applied to cultured cells to elicit a uniform circadian rhythm (Welsh et al. 2004). In cultured fibroblasts, a short duration, high concentration of horse serum (Balsalobre et al. 1998) or even glucose (Hirota et al. 2002) phase aligns the circadian clock among cells, to then evoke a uniform oscillatory clock signal (i.e., *Bmal1*, *Per2* oscillation). Other studies provided evidence that additional cues aside from serum or its components could induce circadian rhythmicity. Glucocorticoids were shown to induce circadian gene oscillation in fibroblasts (Balsalobre et al. 2000), liver cells (Reddy et al. 2007), and vascular smooth muscle cells (McNamara et al. 2001). In human vascular smooth muscle cells and murine aorta, retinoic acid was shown to phase shift the oscillation of circadian clock, evidence that humoral signals may act

to influence the clock (McNamara et al. 2001). Angiotensin II also entrains the circadian clock within vascular smooth muscle cells (Nonaka et al. 2001), an effect dependent on the angiotensin II type 1 (AT1) receptor, suggesting that circadian clock function may be perturbed during renin-dependent hypertension. Though the presence of AT1 receptors in the SCN has been demonstrated (Thomas et al. 2004), it may also be that angiotensin and other vasoactive signals selectively act on the vascular clock, without impinging on central circadian rhythms. Catecholamines may also exert an influence on circadian timing, as norepinephrine and epinephrine can phase advance *Per2* expression in cultured human aortic smooth muscle cells, although, in vivo, peripheral circadian clock rhythms were preserved in dopamine beta-hydroxylase KO mice (Reilly et al. 2008).

Prostaglandins have also been implicated to entrain the circadian clock. In cultured fibroblasts, PGE2 induced rhythmicity of *Per2*, while intraperitoneal injection in mice phase shifted *Per2* expression in heart, liver, and kidney, an effect that was also mimicked by the administration of an agonist to the EP1 receptor (Tsuchiya et al. 2005), a PGE2 receptor subtype important in the regulation of blood pressure (Stock et al. 2001). In another study, a peptide and lipid library were used to screen for circadian activators in a rat fibroblast cell line stably expressing *Per2*-Luc. Endothelin, a potent vasoconstrictor (Yanagisawa et al. 1988) and PGJ2, which has been linked to angiogenesis (Xin et al. 1999), and VEGF signaling (Marx et al. 1998), were identified as inducers of circadian oscillation. In addition, PPARγ which is activated by PGJ2 (Marx et al. 1998; Ricote et al. 1998) may also be an important circadian clock-modifying signal (Wang et al. 2008b). The PPARγ agonist rosiglitazone transactivated the *Bmal1* promoter in transformed endothelial and cancer cell lines, while in aortae of mice, endothelial and smooth muscle cell-specific deletion of PPARγ decreased *Bmal1* oscillation and expression. Future studies are needed to characterize additional agents that modify the circadian clock in vascular smooth muscle and endothelial cells which may yield important therapeutic agents in the control of vascular rhythms.

7.8 Blood Pressure and Circadian Rhythms in Humans

While circadian rhythms are most realized in waking and sleeping, it is also well established that blood pressure exhibits a circadian variation in mammals, including humans (Millarcraig et al. 1978) and mice (Li et al. 1999). Indeed, the elevation of blood pressure, or hypertension, is a major risk factor for cardiovascular disease and death, causing impairments in vasculature, including wall thickening, luminal narrowing, endothelial dysfunction, and wall stiffness which as earlier described are influenced by the circadian clock. In healthy humans, there is a nighttime dip in blood pressure and a rise in the morning hours, while the rhythms are inverted in nocturnal animals such as mice. The circadian rhythm of blood pressure can be abnormal during hypertension, with blood pressure patterns over 24 h non-dipping (O'Brien et al. 1988), extreme dipping (Kario et al. 1996), and reverse dipping

(Kario et al. 2001). These disturbances to the blood pressure rhythm cause increased cardiovascular disease and death (Timio et al. 1995; Chobanian et al. 2003). Thus, having high blood pressure at night, during resting or sleep, can be even more harmful to health than daytime hypertension, which in part may reflect the difficulty in diagnosing the hypertension which would potentially be undetected in daytime office visits. Conversely, controlling the nighttime hypertension is beneficial to health. Perhaps, this was exemplified in the Heart Outcomes Prevention Evaluation Study Investigators (HOPE trial). In this trial, the angiotensin-converting enzyme (ACE) inhibitor ramipril had significant effects on the reduction in the rates of death, myocardial infarction, and stroke (Yusuf et al. 2000). While ramipril did not significantly lower blood pressure during office visits (daytime), a subsequent substudy revealed that ramipril did lower blood pressure at night (Svensson et al. 2001).

Aside from the rhythm in blood pressure, the time of day or night at which antihypertensive drugs are actually administered, called chronotherapy, also differentially influences blood pressure control (Lemmer 1996, 2006). A calcium channel blocker (CCB) (which reduces blood pressure by dilating blood vessels and reducing heart contractility) was shown to cause a greater or improved blood pressure lowering effect on nighttime blood pressure when given at 8 PM when compared to an 8 AM administration. Also, when given at nighttime, the CCB effect to lower nighttime blood pressure also restored the circadian rhythm in blood pressure (Portaluppi et al. 1995). Similarly, the administration of an alpha adrenergic antagonist (which dilates small arterioles) prior to sleeping improved lowering of blood pressure in hypertensive patients (Hermida et al. 2004). Thus, the restoration of circadian variability in blood pressure appears to be an important factor in the control of cardiovascular disease. The topic of chronotherapy in the treatment of hypertension is explored in detail in Chap. 12.

There are numerous control mechanisms involved in blood pressure regulation having discrete tissue specificity. The medulla of the brain controls blood pressure through release of sympathetic and parasympathetic drive to the heart, kidneys, and vasculature. Catecholamines, the product of sympathetic drive, are misregulated in experimental models where rhythm in blood pressure and activity is disrupted (Curtis et al. 2007; Mahapatra et al. 2005; Wang et al. 2008b). In addition to autonomic drive, central clock-modifying signals may also play a role in blood pressure control. Vasoactive intestinal peptide (VIP) modulates central circadian clock function (Albers et al. 1991), impacts vascular function (Ignarro et al. 1987), and is suppressed in hypertensive patients (Goncharuk et al. 2001). The VIP receptor, VPAC2, which has also been implicated in circadian rhythm regulation (Aton et al. 2005; Harmar et al. 2002), is located in the vascular wall (Grant et al. 2006). Melatonin, a peptide secreted from the pineal gland which entrains the SCN circadian clock, has also been implicated in blood pressure (Liu et al. 1997; Van Reeth et al. 1997). In hypertensive patients with impaired circadian variation in blood pressure, melatonin supplementation can improve the lowering of nocturnal blood pressure (Scheer et al. 2004). It remains unclear where these actions occur as melatonin receptors are found not just in the brain, but are also

expressed in the cardiovascular system (Dubocovich and Markowska 2005; Ekmekcioglu et al. 2001; Viswanathan et al. 1990) (see Chaps. 4 and 8 for more information on melatonin in the brain and heart, respectively).

7.9 Insights from Animal Models: The Circadian Clock in Blood Pressure Regulation

Basic science studies have further implicated the circadian clock in the control of circadian blood pressure. In mice, the genetic disruption of the core components of the circadian clock (*Bmal1*, *Clock*, *Npas2*, *Per*, and *Cry*) influences the circadian rhythm in baseline blood pressure. *Cry1*/*Cry2*-KO mice are hypertensive in the daytime (a time when blood pressure falls in rodents) relative to wild-type mice (WT) in conditions of constant darkness, abolishing the circadian variation in blood pressure, which rises in WT mice at night during the activity period but remains unchanged in the CRY-deficient mice (Masuki et al. 2005). The ultimate effect is that CRY-deficient mice have higher blood pressure during the rest period and exhibit fairly normal blood pressure in the activity period. *Bmal1*-KO mice also lose the circadian variation in blood pressure, but this is due to a decreased blood pressure during the activity period (Curtis et al. 2007) contrast from the higher blood pressure observed in the CRY-deficient mice. The effect of BMAL1 to regulate blood pressure seems to be independent of the endothelial layer of blood vessels, as the overall blood pressure rhythm remains intact in mice with endothelial-specific deletion of *Bmal1* (EC-*Bmal1*-KO) (Westgate et al. 2008). These endothelial KO mice do exhibit a reduction in blood pressure at discrete times within the activity phase, but the effect is modest and not observed in the resting phase. *Npas2*-mutant mice retain blood pressure rhythm, but the mice do have a lower blood pressure. Similarly, mutation of Clock in mice also causes only subtle dampening of blood pressure in LD as demonstrated in two background strains of mice, the C57BL/6 (Curtis et al. 2007) or Jcl/Icr (Sei et al. 2008), which may reflect the influence of the light cycle conditioning in these mice and the functional redundancy of BMAL1 to bind either CLOCK or NPAS2 (Debruyne et al. 2006; DeBruyne et al. 2007).

Other signals have been shown to interact with the circadian clock to modulate the circadian variation in blood pressure. Mice with targeted deletion of PPARγ in the endothelium (EC-*Pparg*-KO) are phenotypically similar to EC-*Bmal1*-KO mice with regard to blood pressure (Westgate et al. 2008) (Wang et al. 2008b). EC-*Pparg*-KO mice have lower blood pressures at night, and normal blood pressures during the day; however, rhythm remains largely intact different from the complete ablation of rhythm observed in *Bmal1*-KO mice. However, smooth muscle cell disrupted PPARγ mice (SMC-*Pparg*) are different from the endothelial KOs. SMC-*Pparg* mice have higher blood pressure in the daytime and trend to a lower blood pressure at night, while locomotor rhythm remains intact in

PPARγ-deficient mice (Wang et al. 2008b). This is different from the core clock global KOs that do exhibit impairments in locomotor function, but may suggest that circadian inputs into blood pressure regulation are not solely the result of central clock or activity rhythms. These same studies showed that PPARγ exhibits a direct interaction with BMAL1. Agonist activation of PPARγ increases aortic BMAL1 expression and, correspondingly, loss of PPARγ in the aorta of both EC and SMC-PPARγ-KO mice leads to reduced expression of the core circadian clock components.

EC-*Bmal1*-KO and EC-*Pparg*-KO mice retain overall blood pressure rhythm, but this does not necessarily abrogate a role for the endothelial clock in the regulation of blood pressure rhythm. Circadian rhythm is robust and resistant to perturbation to individual clock components, which may in part occur through redundancy, networking (Baggs et al. 2009), and also intercellular coupling (Liu et al. 2007) of the circadian clock mechanism. Many of the current studies extrapolated effects from the aorta or large arteries that do not control blood pressure. It is the small abundant arterioles that are critical with regard to vasoconstriction and vasodilation to control blood pressure and studies in the so-called resistance vessels are lacking.

7.10 Diabetes, Obesity, and Blood Pressure

While obesity and metabolic dysfunction are major risk factors for the impaired control of blood pressure (DeFronzo and Ferrannini 1991; Reaven et al. 1996), evidence has also implicated the circadian clock. In experimental rodent models of type II diabetes, blood pressure is increased (Carlson et al. 2000; Osmond et al. 2009). The elevation in blood pressure is also characterized by changes in the circadian variation of blood pressure (Goncalves et al. 2009; Senador et al. 2009; Su et al. 2008). The daytime drop in blood pressure in mice is blunted (not as great a drop) in animals with type II diabetes, suggesting that the circadian clock and its management of blood pressure is impaired. This is also observed in humans, where type II diabetes increases the frequency of non-dipping which is observed in up to 75 % of patients (Czupryniak et al. 2007). Again, the loss of nighttime blood pressure dipping is associated with increased cardiovascular mortality (Holl et al. 1999; Izzedine et al. 2006; Sturrock et al. 2000). In addition to type II diabetes, type I diabetes is also characterized by individuals who exhibit blunted circadian regulation of blood pressure which seems to correlate with declining renal function (Lurbe et al. 1993).

In addition to the effects of diabetes to impair blood pressure rhythms, type II diabetes also affects the circadian regulation of heart rate (Goncalves et al. 2009; Su et al. 2008) and locomotor activity (Su et al. 2008). Other aspects of blood pressure control are also altered in animals with type II diabetes including renal function (Cohen et al. 1996), circulating hormones (Friedman and Halaas 1998; Sinha et al. 1979), autonomic reflexes (Schreihofer et al. 2007), and vascular (Bagi

et al. 2005; Didion et al. 2005; Guo et al. 2005; Kamata and Kojima 1997; Kanie and Kamata 2000) and sympathetic tone (Goncalves et al. 2009). These effects may be intricately related to the rhythms in locomotor activity and locomotor activity is frequently used as a surrogate for the activity of the central clock while lesion of the SCN also disrupts the rhythms of feeding, activity, and blood pressure (Witte et al. 1998). Indeed, mice with type II diabetes exhibit a gross reduction in locomotor activity that accompanies the impaired daily variations in blood pressure. Despite the gross reduction in locomotor activity, the circadian rhythm in activity remains intact. This may be a consequence then of the morbidly obese phenotype of the diabetic mice which may act as the primary constraint on activity rather than an alteration of the central clock. Consistent with the idea that these effects are central clock independent, expression of circadian clock genes is unaltered in the SCN of mice with type II diabetes (Kudo et al. 2004). However, the oscillation of circadian genes in the periphery, isolated blood vessels, and the liver is impaired in diabetic mice (Kudo et al. 2004; Su et al. 2008). The link between type II diabetes and altered circadian timing is further complicated by the ability of the circadian clock to influence metabolism and ultimately obesity and diabetes. *Clock*$^{\Delta 19}$ mice exhibit altered feeding patterns leading to obesity and insulin resistance (Turek et al. 2005). Furthermore, in both humans and rodents, polymorphisms of the *Bmal1* gene are associated with increased incidence of type II diabetes and hypertension (Woon et al. 2007). These results support the concept that type II diabetes promotes asynchrony of the peripheral and central circadian clocks and also that this asynchrony may contribute significantly to cardiovascular disease (see Chap. 5 for more information on the role of the clock in metabolism, obesity, and diabetes).

7.11 Angiotensin Influences on the Circadian Rhythm in Experimental Hypertension

Animal models of renin-angiotensin system (RAS)-induced hypertension experimentally reproduce an important manifestation of human hypertension. The renin-angiotensin-aldosterone system produces renin which cleaves angiotensinogen to angiotensin I, which is then converted to angiotensin II (AngII) by the angiotensin-converting enzyme (ACE). AngII exerts its biological effects to constrict blood vessels (to increase blood pressure) and to cause release of aldosterone, which also causes sodium retention to increase blood pressure. There are different animal models with mutations in this pathway that exhibit hypertension and defects in blood pressure circadian rhythm. The TGR(mREN2) rats are transgenic, hypertensive rats that possess an extra copy of the renin gene [TGR(mREN2)27]. TGR (mREN2) rats develop an inverse blood pressure rhythm (inverse/reverse dipper) 6 weeks after birth, transitioning from a blood pressure that peaks at night to a blood pressure that peaks in the day (Witte and Lemmer 1999). Yet, motor activity and

rhythm in the TGR(mREN2)27 rats remain unperturbed (Witte and Lemmer 1999). The spontaneously hypertensive rat (SHR) has a mutation in a genetic locus that is close to the ACE gene (Hilbert et al. 1991; Jacob et al. 1991). SHR rats exhibit a blood pressure rhythm whose peak blood pressure is shifted further toward the resting period (Shimamura et al. 1999). Activity rhythms are also impaired in SHRs. Activity period in SHRs begins 1.5 h earlier than in WKY control rats and their response to light cycle shifts is altered, which may be due to an impairment in vasoactive intestinal peptide signaling (Peters et al. 1994) which is important in circadian locomotor function (Harmar et al. 2002). The SHRs have a single nucleotide polymorphism in the essential circadian clock gene *Bmal1* suggesting an association between the hypertensive phenotype and the *Bmal1* mutation (Woon et al. 2007). SHRs also exhibit enhanced expression of *Clock*, *Bmal1*, and *Per2* expression in heart tissue, albeit their rhythms are retained (Naito et al. 2003). Furthermore, SHRs exhibit enhanced amplitudes in the oscillation of renin, angiotensinogen, ACE, and angiotensin type 1a (AT1a) and type 2 (AT2) receptors in the heart (Naito et al. 2002). Indeed, AngII does have significant effects on the circadian clock in vascular cells. In cultured vascular smooth muscle cells, AngII has potent effects on the circadian clock to induce synchronous circadian oscillation of *Per2* and *Bmal1* expression (Nonaka et al. 2001).

While there are genetic mutants in the RAS pathway, hypertension can additionally be induced by direct administration of the agents. Chronic AngII infusion by osmotic minipump in rats has more robust effects on circadian blood pressure (Sampson et al. 2008) relative to the RAS-defective SHRs. Exogenous administration of AngII abolishes the circadian rhythm of arterial pressure in a gender-independent manner and further causes a modest reverse dipper phenotype in female rats, reminiscent of the response of TGR(mREN2) (Witte and Lemmer 1999) rats. With regard to effects on locomotor rhythm, though exogenous administration of AngII directly to SCN brain slices does stimulate neuronal depolarization (Brown et al. 2008), behavioral rhythm remains intact in AngII infused mice in the face of the profound effects on blood pressure rhythm (Sampson et al. 2008). These results suggest that blood pressure regulation is more complex and cannot be explained solely by changes in activity.

Other mice with targeted genetic disruption to components of the RAS signaling pathway largely retain the baseline rhythm in blood pressure; however, impairments emerge during conditions of experimental hypertension. While *Ace*-KO mice maintain blood pressure rhythm, after administration of a high salt diet, the amplitude of the rhythm is enhanced in *Ace* heterozygotes and blunted in *Ace* KO relative to WT mice (Carlson et al. 2002). Similarly, in AT1a receptor (*Agtr1a*)-KO mice blood pressure rhythm is preserved, but fructose feeding increases the difference in blood pressure between night and daytime (Farah et al. 2007). Again, in *Agtr1a*-KO mice, 5 days of high salt diet abolishes the normal light/dark BP rhythm in *Agtr1a*-KO mice (Chen et al. 2006). This may reflect AT2 receptor expression in kidney or blood vessels, but also relate to brain AT2 receptor expression. This is based on evidence showing that adenoviral transduction of the AT2 receptor in the rostral ventral lateral medulla of rats abolished the circadian variation in blood pressure by

blunting the nighttime spike (Gao et al. 2008). Hypertensive disease may worsen clock function, which may feed forward to further impair the pressor response.

7.12 The Circadian Clock in the Kidney

The kidney is the major organ for long-term blood pressure regulation and is also under circadian regulation. In non-dipping human hypertensive patients, the normal circadian pattern of sodium excretion is blunted in concert with blood pressure (Dyer et al. 1987). Sodium restriction can convert non-dippers into dippers and alternatively sodium loading attenuates dipping suggesting a vital role for the kidney and fluid volume in this process (Fujii et al. 1999; Higashi et al. 1997; Uzu et al. 1997). This may also reflect activation of the RAS pathway, as sodium restriction is a potent stimulus for the angiotensin, renin, and aldosterone. Thiazide diuretics which promote natriuresis (the excretion of sodium and water) can also restore the nighttime reduction in blood pressure (Fujii et al. 1999). Further evidence for a role of the kidney comes from studies showing that loss of renal function following nephrectomy correlates with impaired circadian variation in blood pressure (Goto et al. 2005). Independent studies have begun to uncover putative molecular targets of the circadian clock in the kidney that account for the rhythmic changes in blood pressure. The Na^+/H^+ exchanger (NHE3) appears to be a target of the circadian clock as its expression in the kidney oscillates with a circadian rhythm and its promoter contains functional E-boxes that participate in the transactivation by BMAL1 and CLOCK (Saifur Rohman et al. 2005). However, the loss of NHE3 in mice does not appear to affect the circadian regulation of blood pressure (Noonan et al. 2005). Similarly, loss of the Na–2Cl–K cotransporter does not affect blood pressure rhythm (Kim et al. 2008a). Interestingly, deficiency of carbonic anhydrase II in mice, which is important in sodium and bicarbonate reabsorption in the kidney, alters the circadian period of locomotor rhythm (Kernek et al. 2006).

A reciprocal relationship has been described between a circadian clock component, the *Per1* gene, and sodium balance (Gumz et al. 2009). In these studies, aldosterone which has been shown to oscillate in plasma (Sei et al. 2008) stimulated *Per1* expression in the kidney medulla in vivo and in vitro, further corroborated by reporter assay studies of transcriptional regulation of the *Per1* promoter. It was also shown that the alpha subunit of the renal epithelial sodium channel, αENaC, was an output of the circadian clock. PER1 regulated the expression of αENaC, while its expression was altered in mice lacking functional *Per* genes and by *Per1* knockdown. These studies further demonstrated that urinary sodium excretion was increased in *Per1*-deficient mice. Interestingly, these observations are consistent with observations in *Clock* mutant mice, which have decreased water intake (Sei et al. 2008). These results suggest that the circadian clock may be important in control of sodium balance, while also acting as a sensor to sodium levels via aldosterone.

In addition, circadian changes in renal function may also be dependent on genes extrinsic to the kidney (i.e., liver) and indeed the expression of a large number of genes that affect renal function is known to oscillate in a circadian pattern including renin/angiotensin, kinins, AVP, and uroguanylin to name but a few (Gross et al. 2000; Hurwitz et al. 2004; Kudo et al. 1998; Scheving and Jin 1999; Tominaga et al. 1992). All of these factors are further influenced by age and salt load (Kimura 2008). The circadian clock in the kidney is discussed in detail in Chap. 6.

7.13 The Circadian Clock and Peripheral Vascular Contractility

The dilation of constricted blood vessels that increases peripheral vascular resistance is a significant target of therapy in hypertension. The vasoconstriction/vasodilation or vascular tone of blood vessels is known to exhibit a circadian variation (Panza et al. 1991). Catecholamines clearly play a part in the circadian tone of blood vessels. Circadian clock-deficient mice (*Bmal1*-KO and *Npas2* mutant) (Curtis et al. 2007) and EC-*Pparg*-KO mice [which have reduced BMAL1] (Wang et al. 2008b) have reduced levels of norepinephrine and epinephrine in plasma at both night- and daytime, consistent with the hypotensive phenotype of the mice. Mice with disruption of chromogranin A, which produces the catecholamine inhibitory fragment catestatin (Mahata et al. 1997), have complete impairment in circadian blood pressure rhythm and reduction in adrenal catecholamine levels (Mahapatra et al. 2005). While catecholamines stimulate beta adrenergic receptors to stimulate heart contractility and kidney renin release and thereby raise blood pressure, β1/β2 adrenergic KO mice exhibit a normal blood pressure rhythm (Kim et al. 2008b). However, mice deficient in dopamine β-hydroxylase, the rate-limiting enzyme in catecholamine production, do have an impaired blood pressure rhythm (Swoap et al. 2004) further evidence for epinephrine and norepinephrine in the circadian variation in blood pressure. Indeed, catecholamine release from nerve fibers localized on arterioles of the circulatory system stimulates α-1 adrenergic receptors to cause vasoconstriction and also elevate blood pressure. Thus, it may be that catecholamines mediate circadian changes in blood pressure through α-1 adrenergic receptors to control vascular resistance as opposed to control of cardiac contractility through the α-adrenergic receptors. This is supported by observations demonstrating that the pressor response to an α1-adrenergic receptor agonist is suppressed in *Cry*-deficient mice (Masuki et al. 2005), and in addition there are alterations in the expression of α-adrenergic receptors in these mice.

Nitric oxide has also been implicated as a control mechanism for the circadian variation in blood pressure. There are three isoforms of nitric oxide synthase, neuronal (nNOS), inducible (iNOS), and endothelial (eNOS), that are responsible for the generation of nitric oxide in higher mammals. Indeed, several lines of evidence suggest that eNOS signaling and endothelial function are impaired in

mice with dysfunctional circadian clocks (Anea et al. 2009; Rudic et al. 2005a; Wang et al. 2008a). NO and the ability of the nitric oxide synthase inhibitor, L-NAME, to increase blood pressure vary with time. L-NAME also decreases the diurnal variation in blood pressure (Witte et al. 1995) and along with other NOS inhibitors can modulate the function of the SCN (Kriegsfeld et al. 1999). Additionally, the concentration of nitric oxide metabolites in the plasma also exhibits circadian variation (Mastronardi et al. 2002). Posttranslational mechanisms regulating eNOS activity are compromised in mice with targeted mutation of the circadian clock, consistent with observations demonstrating that eNOS activity exhibits a circadian variation (Tunctan et al. 2002), which may be a consequence of its phosphorylation state (Anea et al. 2009; Kunieda et al. 2008; Wang et al. 2008a). Moreover, vascular disease is worsened in circadian clock mutant mice when challenged by arterial ligation or vascular injury (Anea et al. 2009). Despite this, the rhythm in blood pressure is retained in eNOS-KO mice (Lemmer et al. 2004; Van Vliet et al. 2003), suggesting that circadian rhythms in blood pressure are either not mediated by eNOS or are compensated through other mechanisms. Similarly, the function of the SCN is normal in nNOS-KO mice (Kriegsfeld et al. 1999).

Another mechanism that might account for a reduction in nocturnal pressure is a loss of endothelial function in the arterioles in the circulatory system. That a circadian rhythm exists in the function of human blood vessels has long been known (Kaneko et al. 1968). Indeed, endothelial function varies according to the time of day (Keskil et al. 1996), and this variation is altered in mice with mutated circadian clocks (Anea et al. 2009; Viswambharan et al. 2007), while the downstream effector response to nitric oxide, through guanylyl cyclase, remains intact (Anea et al. 2009; Viswambharan et al. 2007), consistent with observations in humans which demonstrate that the response to sodium nitroprusside does not vary according to time of day (Panza et al. 1991). Small vessels or arterioles exhibit stiffening in *Bmal1*-KO and *Per*-KO mice (Anea et al. 2010), which may compromise the vasodilatory process important in blood pressure. In individuals with compromised endothelial function which is often accompanied by vascular stiffening, the diurnal variation in blood vessel function is blunted (Shaw et al. 2001). Forearm blood flow is also compromised in non-dipping vs. dipping hypertensives (Higashi et al. 2002). Moreover, hypertension is known to modify the circadian clock in the heart and vasculature (Mohri et al. 2003; Naito et al. 2003; Young et al. 2001). These findings raise important questions as to whether a loss of circadian rhythms causes endothelial dysfunction or if loss of endothelial function results in the disruption of circadian regulation. Indeed, hypertension is known to modify the circadian clock in the heart and vasculature (Mohri et al. 2003; Naito et al. 2003; Young et al. 2001). Furthermore, endothelial dysfunction occurs in arteries from mice with genetic disruption of the circadian clock (Anea et al. 2009; Viswambharan et al. 2007).

Superoxide is a form of extremely reactive oxygen having a free electron, and its generation is a powerful antagonist to the vasculoprotective actions of NO and a frequent cause of endothelial dysfunction in cardiovascular disease states. Blood

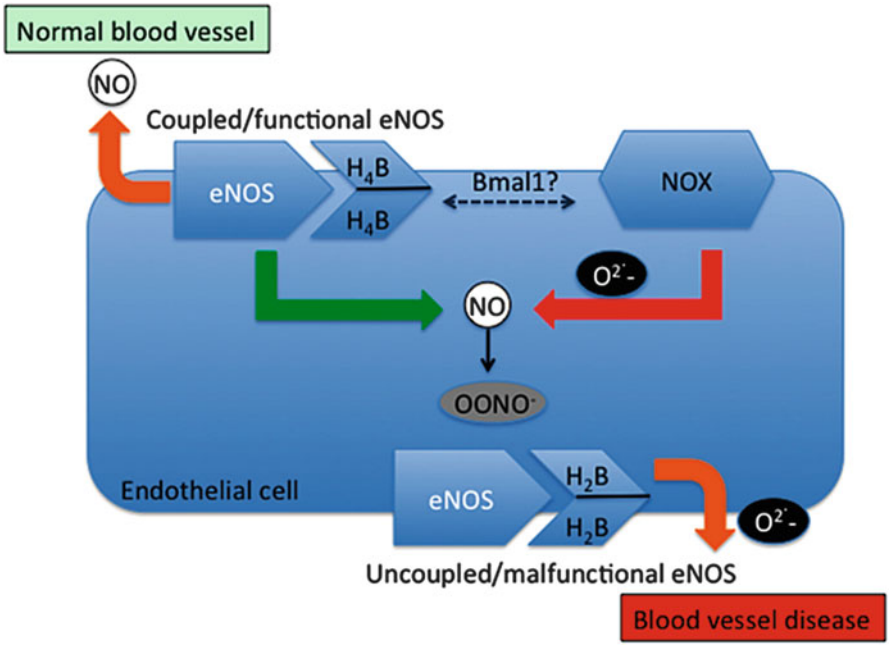

Fig. 7.3 Uncoupled endothelial nitric oxide synthase (eNOS). During healthy/normal conditions, the enzyme eNOS produces NO in part via interaction with tetrahydrobiopterin (H_4B), a cofactor that facilitates its NO-producing activity. Superoxide generated via NADPH oxidase enzymes (NOXes) can oxidize H_4B to H_2B to uncouple eNOS and produce superoxide ($O^{2\bullet-}$) to impair smooth muscle cell dilation and propagate vascular disease. The circadian clock component Bmal1 has been shown to exert an important role in this pathway

vessels from hypertensive animals overproduce superoxide and accompanying ROS which alters vascular tone and induces endothelial dysfunction (Rajagopalan et al. 1996). Increased production of ROS occurs in kidneys and spleen from *Bmal1*-KO mice (Kondratov et al. 2006). Moreover, the levels of superoxide production in brain and neutrophils have been shown to exhibit circadian variation (Baran et al. 2000; Muniain et al. 1991) and superoxide production is increased in *Bmal1*-KO mice (Anea et al. 2012). These data suggested that uncoupling of eNOS from its cofactor BH4 was a source of superoxide production (Fig. 7.3). Increased production of ROS can also disrupt the timing of the circadian clock (Hardeland et al. 2003; Zheng et al. 2007), suggesting a potential mechanism for stimuli that induce ROS production such as high blood pressure or diabetes to disrupt the local circadian clock and promote vascular disease. Another form of oxidant stress, hydrogen peroxide, is also regulated by the circadian clock. These data showed that the enzyme responsible NOX4 was upregulated in *Bmal1*-KO mice and that hydrogen peroxide was also increased in arteries (Anea et al. 2013).

It is evident that the regulation of the circadian clock impinges on multiple mechanisms involved in blood pressure regulation. The hands of the clock may directly touch upon the genes and proteins involved in blood pressure regulation,

i.e., through direct transcriptional control and posttranslational regulation. Perturbations to the circadian clock may occur in a silent manner perhaps influencing only peripheral clocks, i.e., non-dipping hypertension or through central behavioral aberrations, i.e., sleep apnea, sleep duration, and shift work. One possibility is that the genetic components of the circadian clock may act to directly activate or repress such mechanisms in tissues intrinsically important in blood pressure regulation or in tissues extrinsic to the pressor mechanism by release of mediators such as hormones or circulating peptides. Transcriptional activation by the positive limb of peripheral circadian clocks may transactivate genes important in vascular (Rudic et al. 2005b), cardiac (Storch et al. 2002), or renal function (Kita et al. 2002) and these functions can be antagonized by proteins of the negative limb. Future studies are needed to examine if there is an impact of ROS production in vascular function and blood pressure regulation in mice with a disrupted circadian clock as ROSs are established negative modulators (Laursen et al. 1997). Moreover, it will be important to identify the putative signals that act as intermediaries to blood pressure and circadian control.

7.14 Conclusion

Circadian rhythms are innate in terrestrial organisms enabling anticipation of environmental in internal oscillations. The circadian clock in the SCN receives temporal input from the environment, while the circadian clock in cardiovascular system receives temporal input from the internal environment of the body, from cells to organs, from circulating blood and humoral signals. While much insight has been gleaned from animals with genetically engineered disruption of the circadian clock, clock dysfunction also occurs in the absence of genetic mutation. Environmental or behavioral aberrations that directly impact central clock function such as shift work and sleep disorders impinge on cardiovascular homeostasis. In addition, circadian dysfunction may extend beyond these centrally derived anomalies. Circadian clock function deteriorates with age in the cardiovascular system, to impair vascular cell signaling and function. Having such an elaborate role in the control of vascular cell signaling and in the control of daily cycles of endothelial function, blood pressure, and hemostasis, the impact of circadian clock dysfunction is significant when it malfunctions in the long-term to cause disease. Thus, understanding the impact of circadian function in the cardiovascular system may provide new frontiers in our understanding of human disease and medicine.

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Chapter 8
The Cardiac Clock

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Abstract The circadian clock mechanism is integral to the cardiovascular system, underlying rhythmic variations in normal cardiovascular physiology including heart rate, blood pressure, autonomic bias, and cardiac metabolism. This mechanism also plays an important role in cardiovascular disease, influencing the timing of onset of adverse cardiovascular events such as myocardial infarction, ventricular arrhythmia, and sudden cardiac death. Disturbing rhythms adversely affects cardiac physiology and exacerbates heart disease. Moreover, there is emerging evidence that the circadian mechanism plays a role in cardiac sarcomere function by influencing myofilaments, the proteins responsible for cardiac contraction and the largest consumer of energy in cardiomyocytes. Lastly, translational studies on diurnal molecular biomarkers, and on timing of drug therapies (chronotherapy), have opened new opportunities for diagnosing and treating cardiovascular disease. This chapter will review the key roles of the cardiac clock in health and disease and translational applications to benefit patients clinically.

Keywords Circadian • Heart • Cardiovascular disease • Sarcomere • Chronotherapy • Biomarkers

8.1 Introduction

Cardiovascular diseases (CVD) are a leading cause of morbidity and mortality worldwide, with more than 17 million deaths each year (World Health Organization [WHO] 2011). In Canada, the estimated economic burden of CVD is 22.2 billion dollars per year in physician services, hospital costs, lost wages, and productivity

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(Health Canada 2009). The economic impact in the United States is even greater, with an estimated 32.4 billion dollars of national health funding spent directly on the treatment of heart failure patients alone, and this cost is expected to rise (Heidenreich et al. 2011). Thus, there is a great need to advance our understanding of the mechanisms responsible for the pathogenesis and pathophysiology of CVD to aid in the development of new diagnostic and treatment strategies. Circadian rhythms have emerged as a novel translational approach for investigating CVD. This chapter will discuss the role of the circadian clock in (1) normal cardiovascular physiology, (2) heart disease including myocardial infarction, ventricular tachyarrhythmia, and sudden cardiac death, (3) rhythm disruption and heart disease, (4) the cardiac sarcomere, and (5) translational applications including biomarkers and chronotherapy (timing of treatment) for CVD.

8.2 The Circadian Clock and Normal Cardiovascular Physiology

8.2.1 Diurnal Rhythms in Heart Rate

The physiology of humans and other mammals undergoes diurnal variation as an adaptation to the 24-h light/dark or day/night cycle on Earth [as reviewed by Reppert and Weaver (2002), Hastings et al. (2003), and Dibner et al. (2010)]. One key aspect important to the cardiovascular system is the diurnal variation in the heart rate (HR), which is highest during the waking hours and lowest during sleep time. This was first demonstrated in humans by continuous electrocardiography monitoring of patients over 24 h day/night cycles (Millar-Craig et al. 1978). The circadian system plays a role in regulating diurnal HR, as humans maintained under constant darkness continue to display rhythmic variation in HR even in the absence of light (Scheer et al. 1999). Moreover, experimental studies in rats demonstrate that diurnal HR rhythms are under regulation of the central circadian mechanism in the hypothalamic suprachiasmatic nucleus (SCN), as surgical ablation of the SCN abolished these HR rhythms (Saleh and Winget 1977). Potential neural pathways by which the SCN regulates the heart have been demonstrated by the use of retrograde pseudorabies virus tracing techniques (Scheer et al. 2001). Further support for this notion of central regulation comes from studies using *Clock* mutant mice, in which genetic loss of the core circadian mechanism blunts diurnal HR rhythms in radiotelemetry recordings (Sei et al. 2008). Together these studies reveal a diurnal rhythm in HR and underlying regulation by the central circadian mechanism. The circadian clock in the vasculature is further discussed in Chap. 7.

8.2.2 Diurnal Rhythms in Blood Pressure

A second aspect important to the cardiovascular system is the diurnal variation in blood pressure (BP), which peaks in the daytime and decreases at night, as has been demonstrated in humans by continuous monitoring of intra-arterial pressure over 24 h day/night cycles (Millar-Craig et al. 1978). Daily variations in BP rhythms, as with HR, are partly regulated by the central circadian system, as has been demonstrated experimentally. For example, BP rhythms persist in rats housed under circadian conditions (constant darkness) and monitored by free moving radiotelemetry recordings (Takezawa et al. 1994). Conversely, there is a loss of diurnal variation in BP in rats in response to surgical ablation of the SCN (Janssen et al. 1994). Further support for central regulation of BP rhythms comes from genetic studies using circadian clock mechanism (*Bmal1* and *Clock*) mutant mice which display blunted diurnal BP rhythms (Curtis et al. 2007). Collectively, these studies demonstrate a diurnal rhythm in BP rhythm that is regulated by the circadian mechanism.

8.2.3 Diurnal Biases of the Autonomic Nervous System

Diurnal variations in the autonomic nervous system are relevant to the cardiovascular system. For example, plasma catecholamine levels of norepinephrine and epinephrine in humans increase during wake time, mirroring the increasing BP and HR rhythms at that time (Richards et al. 1986). Moreover, cardiac sympathovagal balance exhibits diurnal variation, with sympathetic biases predominating over parasympathetic during the day time and vagal dominance occurring during sleep (Furlan et al. 1990). A role for circadian regulation of autonomic balance has been indicated by experimental rat studies, in which light stimuli increased sympathetic and suppressed vagal nerve activity in SCN-intact but not in SCN-lesioned animals (Niijima et al. 1993). Distinct sympathetic and parasympathetic neurons within the SCN allow for differential activation based on the time of day (Buijs et al. 2003).

8.2.4 Diurnal Aspects of Cardiac Metabolism

The heart exhibits diurnal rhythms in metabolism that are in part regulated by the circadian system. Experimental rat studies demonstrate changes in glucose oxidation across the diurnal cycle (Young et al. 2001a). Moreover, there is preferential incorporation of oleate into phospholipids, diacylglycerides, and triacylglycerides in rat hearts isolated during sleep time (Zeitgeber Time ZT06) as compared to wake time (ZT18) (Durgan et al. 2007). The role of the cardiac clock in metabolism has been elegantly investigated using cardiomyocyte-specific clock mutant mice

(CCM) that overexpress a mutated CLOCK protein in the heart (Durgan et al. 2006). Under normal conditions the response of the heart to fatty acids is the greatest during the animal's wake time as compared to sleep time, and this response is impaired in CCM mice (Durgan et al. 2006). Diurnal variations in cardiac non-oxidative fatty acid metabolism (triglyceride turnover, lipolysis) are also altered in CCM mice (Tsai et al. 2010). Moreover, circadian regulation of the hexosamine biosynthetic pathway produces rhythmic protein *O*-GlcNAcylation in wild type but not CCM hearts (Durgan et al. 2011). Circadian regulation of metabolism is important for cardiac responses to workload in the day vs. the night, flexibility in substrate utilization, mitochondrial function, and diurnal cardiac gene expression (Bray et al. 2008).

8.2.5 *Discovery of the Molecular Clock in the Cardiovascular System*

A molecular mechanism underlies diurnal cardiac physiology [e.g., as reviewed by Martino and Sole (2009), Durgan and Young (2010), and Paschos and FitzGerald (2010)]. The first discovery of rhythmic cycling of circadian mechanism genes in the heart, specifically *Clock*, *Bmal1*, *Cry1*, *Cry2*, *Per1*, *Per2*, and *Per3*, as well as the output genes *Dbp*, *Hlf*, and *Tef* was demonstrated in rat heart by polymerase chain reaction (PCR) (Young et al. 2001b). It was subsequently shown that cardiovascular tissue explants contain functional circadian clocks, using rat heart and vascular tissues which display rhythmic per-1 luciferase activity that persists for 3–12 circadian cycles (Davidson et al. 2005). Importantly, rhythmic gene expression (*Per1*, *Per2*, and *Bmal1*) has been demonstrated in human heart by PCR (Leibetseder et al. 2009). Notably, because humans are diurnal and rodents are nocturnal, the human circadian genes cycled antiphase to the rodent patterns, as anticipated.

8.2.6 *Clock-Controlled Output Genes of the Heart and Vasculature*

Although the earlier studies noted above identified core circadian mechanism genes cycling in cardiovascular tissues, demonstration that the clock mechanism also regulated expression of other output genes required the advent of global microarray gene expression technology. The first large-scale microarray study revealed that 462 of 5120 heart genes analyzed (~9 %) are rhythmically expressed in murine heart under circadian conditions (constant darkness) by Affymetrix high-density microarrays and bioinformatics analyses (Storch et al. 2002). Since humans live in a diurnal (24-h day/night) environment, and since translational applications

necessitate the understanding of molecular mechanisms in the diurnal environment, we next demonstrated that 1634 of 12,488 genes analyzed (~13 %) are rhythmic under regular 24-h light:dark conditions by Affymetrix microarrays and bioinformatics analyses (Martino et al. 2004). Rhythmic expression of core circadian genes and output genes has been demonstrated in the vasculature as well; 307 of 7000 genes interrogated (~4 %) are rhythmic in murine aortae with the main cassettes belonging to carbohydrate and lipid metabolism, protein processing, vascular structure, and integrity (Rudic et al. 2005). Rhythmic expression of cardiovascular output genes involves several co-regulatory mechanisms. First, the cardiomyocyte circadian mechanism plays a role, as mutation of the CLOCK protein specifically in the heart alters diurnal cardiac gene expression (Bray et al. 2008). Second, a critical role of the neural input from the SCN has been demonstrated as cardiac clock (*Per1*, *Per2*, and *Bmal1*) rhythms are lost in SCN-lesioned mice and cannot be restored by parabiosis to intact SCN animals (Guo et al. 2005). Third, exogenous administration of the pineal hormone melatonin modulates expression of the circadian genes *Bmal1* and *Per2* in rat heart (Zeman et al. 2009) and expression of 233 of 15,247 murine cDNA clones (~1.5 %) on microarray and bioinformatics analyses (Anisimov et al. 2002). In addition, diurnal gene expression in the murine heart can be altered by restricted feeding (Damiola et al. 2000). Light phase restricted feeding can also alter whole body substrate utilization in mice, energy balance, and the circadian/metabolic gene expression phase relationships between the heart and other peripheral tissues (Bray et al. 2012). Rhythmic expression underlies our diurnal physiology, helping to ensure that processes occur at the appropriate time of day or night.

8.3 Circadian Clock and Timing of Onset of Adverse Cardiovascular Events

8.3.1 Diurnal Variation in Timing of Onset of Myocardial Infarction

Timing of onset of myocardial infarction (MI, heart attack) exhibits a diurnal rhythm, as the incidence of patients presenting with ST segment elevated MI (STEMI) is greatest early in the morning as compared to any other time of day or night (Muller et al. 1985). The window of greatest incidence of MI onset narrows to the first 3 h of awakening, which corresponds to ~6:00–9:00 AM, when patient wake time is considered (Willich et al. 1991). Almost 30 years since the initial observations, a diurnal rhythm in the timing of onset of MI is still reported despite lifestyle and therapeutic changes (Kanth et al. 2013).

8.3.2 Diurnal Variation in Severity of Myocardial Infarction

In addition to timing of onset of MI, there is also a diurnal variation in disease severity post-MI. Experimentally, wild-type mice displayed a time-of-day induced variation in infarct size that peaks at the sleep to wake transition time (ZT12); this diurnal variation is lost in CCM mice that have a mutated CLOCK protein in the heart (Durgan et al. 2010). Diurnal variation in severity of MI was subsequently demonstrated in human patients presenting with STEMI, using blood creatine kinase (CK) and troponin I (TnI) concentrations as surrogate markers of infarct size (Suarez-Barrientos et al. 2011). Though the largest infarcts generally occur during sleep or in early morning (Arroyo Ucar et al. 2012), there is some discrepancy as to the exact timing of the largest infarct size in human patients treated by reperfusion post-MI (Reiter et al. 2012). Despite this discrepancy, it is evident that a temporal component influences severity of MI, with obvious implications for patient treatment and outcome.

8.3.3 Diurnal Variation in Inflammatory Responses Relevant to MI

A key factor that can contribute to the diurnal variability in cardiac remodeling and especially scar formation following MI is the immune system, as both cellular and humoral responses have been reported to be under circadian control. For example, 1403 of 17,308 genes analyzed (~8 %) are rhythmically expressed in isolated macrophages by microarray and bioinformatics analyses, including genes important for cytokine production (Keller et al. 2009). As a corollary to this, rhythm disruption alters inflammatory responses and decreases survival following lipopolysaccharide (LPS) challenge in mice subjected to a chronic jet lag protocol, consistent with circadian regulation of the immune system (Castanon-Cervantes et al. 2010). Moreover, the adaptive arm of the immune system is also under circadian regulation, as T-cell proliferative responses exhibit circadian variation (e.g., following T-cell receptor activation of isolated T-cells in vitro and challenge of mice with bone marrow-derived dendritic cells loaded with class I-restricted ovalbumin in vivo) (Fortier et al. 2011). Cytokines are also regulated in part by the circadian mechanism as genetic ablation of the core clock mechanism component REV-ERBα, or pharmacological stimulation of REV-ERBα, alters LPS-induced cytokine expression in mice and in isolated macrophages (Gibbs et al. 2012). Clearly, there is an emerging role for the circadian mechanism in regulating immune system function, including aspects crucial to cardiac remodeling post-MI. The circadian clock in the immune system is covered in more detail in Chap. 9.

8.3.4 Diurnal Variation in Metabolism Relevant to MI

During the early ischemic period post-MI, cardiomyocytes shift metabolic requirements from a high reliance on fatty acids to more oxygen-efficient processes for energy production to maintain cardiac function and limit cell death [as reviewed by Jaswal et al. (2011)]. The importance of the circadian mechanism in metabolic adaptations to ischemia was demonstrated using circadian mutant *Per2* mice, which had increased infarct size attributed to an impaired ability to utilize carbohydrates and upregulate glycolytic enzymes, as compared to wild-type littermates (Eckle et al. 2012). Furthermore, ischemic *Per2* mutant hearts exhibit altered expression of genes important for lipid metabolism by microarray gene expression profiling and increased intra-myocardial monounsaturated fatty acid content by nuclear magnetic resonance spectroscopy (Bonney et al. 2013b). Collectively, these studies demonstrate a role for the circadian mechanism, and especially the core protein PER2, in regulating early metabolic responses crucial for outcome post-MI. Indeed, in light of these findings PER2 has been suggested as a potential therapeutic target for the treatment of MI (Eckle et al. 2012) [reviewed by Bonney et al. (2013a)].

8.3.5 Diurnal Variation in Timing of Onset of Ventricular Tachyarrhythmia

Adverse cardiovascular events in addition to MI have also been shown to exhibit diurnal variation. There is a morning peak in ventricular tachyarrhythmias (VT) in humans, as recorded by implanted cardioverter defibrillators (Tofler et al. 1995). There is also a diurnal rhythm in the ventricular refractory period, which exhibits the greatest change within 2 h of waking (Kong et al. 1995). Experimental murine studies have revealed potential underlying mechanisms for these observations. For example, the potassium channel-interacting protein 2 (KChIP2), a subunit of a potassium ion channel required for normal cardiomyocyte repolarization, is under the control of the circadian regulated *kruppel-like factor 15 (Klf15)* output gene (Jeyaraj et al. 2012). Also, the ion channel component *Scn5A*, a subunit of a cardiac sodium ion channel critical for generation of action potentials, is regulated by the circadian mechanism as identified through the use of an inducible strain of cardiomyocyte-specific *Bmal1* knockout mice that displayed enhanced susceptibility to arrhythmias (Schroder et al. 2013). Thus, there is a previously undiscovered diurnal nature to cardiac electrophysiology, which can provide new insights for understanding the pathophysiology of arrhythmias.

8.3.6 Diurnal Variation in Sudden Cardiac Death

A diurnal rhythm in sudden cardiac death (SCD) has also been observed in humans, with greater prevalence during the early waking hours (7:00–11:00 AM), as compared to later in the day or at night, based on retrospective analyses of mortality records from the Massachusetts Department of Public Health (Muller et al. 1987) and the Framingham Heart Study (Willich et al. 1987). This diurnal variation in SCD exhibits a similar pattern to the timing of onset of MI, and VT. Moreover, SCD likely has similar underlying pathophysiologic factors contributing to it as well, as described below.

8.3.7 Underlying Circadian Mechanisms

Several studies have helped define circadian mechanisms underlying diurnal cardiac physiology in healthy humans, through the use of a forced dyssynchrony (FD) protocol that uncouples behavioral and circadian contributions to physiological processes. In patients subjected to this protocol, the autonomic response to exercise (a behavioral stressor) differs across the circadian cycle with greatest sympathetic, vagal, and catecholamine reactivity during the early waking hours (Scheer et al. 2010). Moreover, platelet activation surface markers glycoprotein (GP) IIb-IIIa, GPIb, and P-selectin exhibit a circadian rhythm that peaks in the morning at 8:00–9:00 AM, independent of behavior (Scheer et al. 2011). Plasminogen activator inhibitor-1 (PAI-1), a key inhibitor of fibrinolysis, also exhibits a circadian rhythm that peaks in the early waking hours and is independent of behavioral rhythms (Scheer and Shea 2014). This is consistent with earlier studies that demonstrated a diurnal rhythm in platelet aggregability that had the greatest increase between 6:00 and 9:00 AM compared to any other time of the day in isolated platelets stimulated by adenosine diphosphate or epinephrine (Tofler et al. 1987). These findings of underlying circadian rhythms in normal healthy human physiology are all factors that likely also contribute to CVD, as supported by experimental murine studies. For example a diurnal rhythm in thrombotic responses has been demonstrated in wild-type mice but not *Clock* mutant mice following photochemical-induced vascular injury (Westgate et al. 2008). Furthermore, platelet activity is blunted in *Clock* mutant mice compared to wild-type littermates (Ohkura et al. 2009). Taken together, these studies shed new light on our understanding of circadian rhythms in normal human physiology and in the pathophysiology of heart disease.

8.4 Circadian Rhythm Disruption

8.4.1 Disturbed Rhythms and Heart Disease

The circadian mechanism underlies healthy cardiac physiology and disruption is associated with heart disease. For example, cardiomyopathic hamsters exhibit increased early mortality following weekly 12 h phase shifts of a 12 h light (L):12 h dark (D) cycle as compared to non-shifted cardiomyopathic littermates (Penev et al. 1998). Moreover, mice with pressure overload-induced cardiac hypertrophy have exacerbated cardiac remodeling and accelerated progression to heart failure when subjected to diurnal rhythm disruption (10 h L:10 h D cycle) as compared to normal 12 h L:12 h D cycle; these adverse outcomes can be prevented by restoring the normal diurnal environment (Martino et al. 2007a). Furthermore, circadian mutant *tau*/+ heterozygote hamsters develop dilated cardiomyopathy when housed in a 24 h diurnal environment that is out of sync with their 22 h mutated circadian mechanism (Martino et al. 2008). Though generally not yet appreciated, failure to entrain to an L:D cycle out of synchrony with the intrinsic cellular circadian period inhibits organ growth, renewal, and repair, leading to organ pathology.

8.4.2 Shift Work and Heart Disease

Rhythm disturbance alters normal diurnal physiology and can have profound effects on our health. For example, shift workers do not show permanent adaptation to the work schedule resulting in misaligned circadian and behavioral rhythms as determined through analysis of plasma melatonin rhythms (Folkard 2008). Additionally, shift workers exhibit altered cardiac autonomic profiles on 24 h electrocardiogram recordings (Furlan et al. 2000) and BP profiles on 24 h noninvasive ambulatory recordings (Chau et al. 1989). Shift work is associated with increased risk of coronary heart disease as demonstrated in a prospective study of nurses (Kawachi et al. 1995). An association between shift work and metabolic syndrome (a risk factor for CVD) also exists as determined by an analysis of 27,485 subjects enrolled in the Västerbotten intervention program (Karlsson et al. 2001). Furthermore, increased prevalence of MI in shift workers has been demonstrated by a meta-analysis of 34 studies comprising prospective, retrospective, and case–control studies (Vyas et al. 2012).

8.4.3 Sleep Disorders and CVD

Patients with sleep disorders (e.g., sleep apnea) have disturbed diurnal physiology, including elevated nocturnal BP (Tilkian et al. 1976). Obstructive sleep apnea (OSA) is associated with altered HR and BP dynamics and increased sympathetic activity compared to control subjects (Narkiewicz et al. 1998). Furthermore, sleep disruption is associated with the development of hypertension, a major risk factor for CVD, as demonstrated by a prospective study of patients with sleep-disordered breathing over a 4-year period (Peppard et al. 2000). Disruption can also impact disease pathophysiology; OSA patients presenting with STEMI had reduced survival compared to patients without OSA by 18 months after the primary event (Lee et al. 2011). For further studies on sleep disruption and heart disease, the reader is referred to several excellent reviews (Bradley and Floras 2009; Drager et al. 2013; Kohler and Stradling 2010; Hayes et al. 2009). Thus, rhythm disruption is an important contributing factor to the genesis and progression of CVD, with implications for humans subjected to diurnal disturbances as a part of their everyday environment. Circadian rhythms and sleep are covered in more detail in Chap. 3.

8.5 The Circadian Clock and the Cardiac Sarcomere

Although there is a vast and growing body of knowledge regarding the molecular nature of cardiac contractility, relatively little is known about how circadian rhythms influence myofilament activity. As described below, there has been a flurry of studies shedding new understanding on how the circadian clock may influence cardiac myofilaments during sarcomere assembly, signaling, and remodeling.

8.5.1 Myofilaments: Overview of Structure and Function

Cardiac contractility is driven by intracellular calcium binding to myofilaments and is regulated by cell signaling and maintenance of the contractile units of cardiac muscle. In cardiac muscle the basic contractile filaments are composed of specialized myofilament proteins which form the cardiac sarcomere (Fig. 8.1). In the sarcomere, filamentous actin and myosin are arranged in parallel to form thin and thick filaments, respectively. Actin–myosin binding forms a cross-bridge which rotates toward the center of the sarcomere, sliding the thin filaments past the thick filaments, shortening the sarcomere, and producing the contractile motion. The sarcomeric proteins troponin and tropomyosin regulate actin–myosin interaction, while these and other myofilament proteins including myosin binding protein C and the myosin light chains influence actin–myosin binding. There is emerging evidence to suggest that elements of the cardiac Z-disc such as α-actinin, CapZ, and

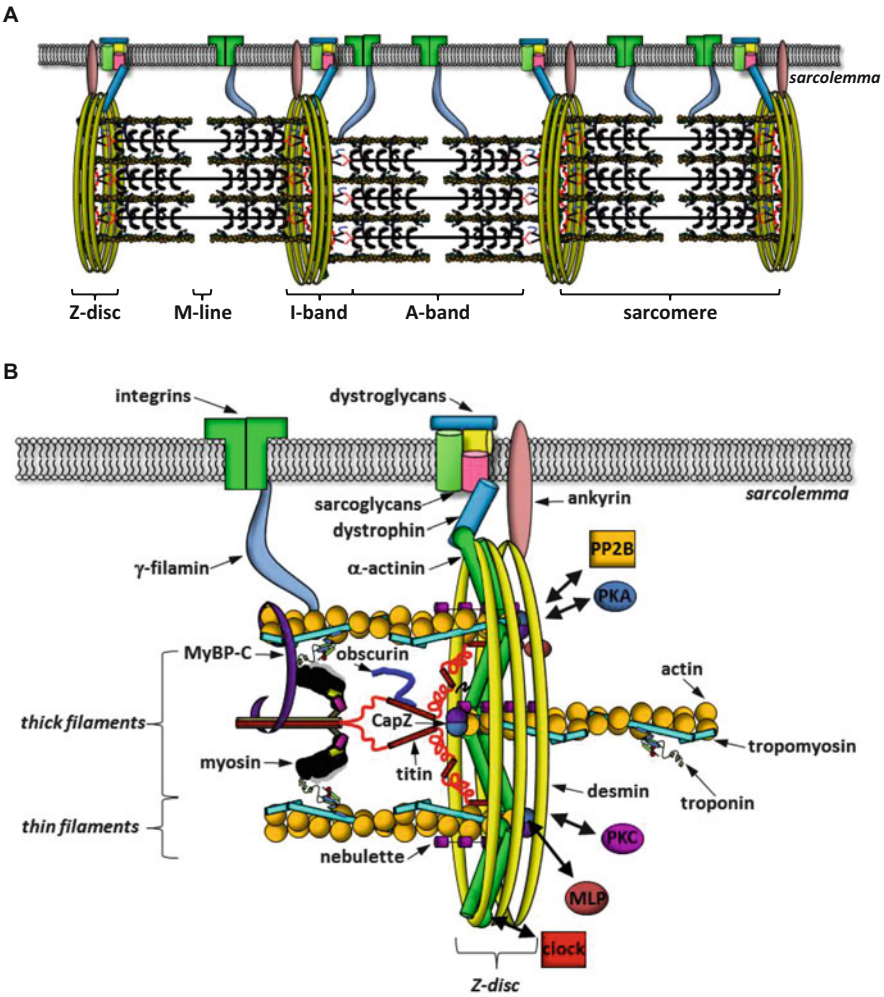

Fig. 8.1 Schematic representation of cardiac myofilaments. (**a**) Model of cardiac sarcomeres showing subcellular domains. (**b**) Expanded image of cardiac sarcomere focusing on anchoring of myofilament proteins to Z-disc. Several key proteins are represented, as are molecular signals that translocate between Z-discs and other subcellular structures

desmin are more than passive structural elements that link adjacent sarcomeres and are instead active regulators of cardiac function (Sequeira et al. 2013; Pyle and Solaro 2004; Frank and Frey 2011). Other components such as titin, which links the Z-disc to the M-line to stabilize myosin and control the length of the sarcomere, are also significant contributors to passive tension of the sarcomere, which in turn influence myocardial contractility [as reviewed by Gautel (2011), Kruger and Linke (2011), and LeWinter and Granzier (2010)].

Our knowledge of the sarcomere is continually expanding. The association of numerous signaling molecules with myofilament proteins and their movement to and from the nucleus with alterations in the physiological milieu have implicated cardiac sarcomeres as drivers of gene regulation [as reviewed by Kruger and Linke (2011), Frank and Frey (2011), Sequeira et al. (2013), and Buyandelger et al. (2011)]. Moreover, the tight coupling of energy metabolism with muscle contractility is not a unidirectional path in which energy supply determines myofilament activation. For example, changes in sarcomeric function are communicated to mitochondria to alter intracellular metabolism to meet the changing demands of the cell (Yaniv et al. 2008). In light of these advances in knowledge the sarcomere is now considered a network of contractile elements capable of signaling to and influencing other cellular compartments to regulate heart function.

8.5.2 The Circadian Clock and the Sarcomere

The CLOCK protein is hypothesized to act as a sensor of myofilament function, as it colocalizes with α-actinin in the Z-disc and translocates to the nucleus upon α-adrenergic stimulated contractile activity (Qi and Boateng 2006). Nuclear shuttling of CLOCK was directly dependent on the changes in myofilament activity, as both L-type calcium channel antagonism and cross-bridge inhibition by butanedione monoxime prevented CLOCK translocation (Qi and Boateng 2006). Moreover, it has been proposed that CLOCK translocation to the nucleus could initiate the CLOCK/BMAL1 complex formation to influence the gene expression in response to changes in cardiac contractility (Qi and Boateng 2006). It is important to note that the presence of CLOCK within the Z-disc has recently been disputed. Wang and colleagues could not confirm CLOCK as an active component of Z-bands and suggested that the earlier findings by Qi and Boateng were due to cross-reactivity of the CLOCK antibody with α-actinin (Wang et al. 2012). While the non-specificity of the CLOCK antibody used by Qi and Boateng may explain the Z-disc staining, it is difficult to explain the apparent nuclear translocation of α-actinin if this is the protein recognized by the antibody. Thus collectively, these data represent both the first report of a circadian element to be directly associated with the cardiac sarcomere and evidence of a link between myofilament function and the circadian mechanism in the heart.

8.5.3 Assembly and Turnover of the Sarcomere

The sarcomere is not a static structure, but dynamic, undergoing constant turnover and remodeling in response to development, physiological drive, and pathological stress. Whereas skeletal muscle myofilaments can be replaced or repaired during periods of reduced activity, cardiac myofilaments must be restructured while active.

Despite this challenging environment, the integration and exchange of proteins within the cardiac sarcomere occur continuously (Willis et al. 2009; Barany and de Tombe 2004). Assembly and turnover of sarcomeric proteins are essential to preserving cardiac function, yet despite its importance, little is known about the mechanisms responsible (Willis et al. 2009). It is thought that circadian rhythms play a role in regulating events such as growth and renewal; these may preferentially occur during periods of low cardiac activity such as during sleep (Martino and Sole 2009). However, the underlying mechanisms for temporal preferences in restructuring have not yet been determined.

8.5.4 The Cardiac Clock and Sarcomere Structure/Function

Loss of the circadian clock function has been reported to disrupt sarcomere structure, alter myosin heavy chain mRNA isoform expression, shift titin isoform composition, and lead to dilated cardiomyopathy (DCM), as demonstrated in mice homozygous for a mutation in the core circadian mechanism gene *Bmal1*$^{-/-}$ (Lefta et al. 2012). Moreover, structural and functional impairment of skeletal muscle myofilaments has been demonstrated by the disruption of CLOCK and BMAL1 in genetic knockout mice, further supporting a role of the circadian clock in sarcomere and myofilament structure and function (Andrews et al. 2010).

8.5.5 Circadian Signaling and Myofilaments

Differential control of myofilament function throughout the day–night cycle has not yet been directly investigated, but several studies have produced evidence to strongly support this concept. In their investigation of gene expression under control of the cardiomyocyte circadian clock, Bray et al. identified signal transduction genes to comprise the largest group of genes regulated by the cardiomyocyte clock (Bray et al. 2008). Specifically mentioned was the ubiquitous cAMP-dependent protein kinase (PKA), which is well known to affect myofilament function through the phosphorylation of several sarcomeric proteins. Moreover, diurnal variation in β-adrenergic receptor activation, a transmembrane receptor that is coupled to PKA activation, has been demonstrated in cardiac myocytes (Collins and Rodrigo 2010; Collins et al. 2013). Combined, these circadian variations in the β-adrenergic receptor-PKA cascade offer a powerful myofilament regulatory pathway that could be regulated by the cardiac circadian mechanism.

8.5.6 Circadian Action Potentials and Myofilament Function

Cardiac myofilament function is highly dependent on action potentials, in particular calcium entry through L-type calcium channels. Expression and current densities of L-type calcium channels demonstrated circadian variation (Collins et al. 2013; Ko et al. 2010) that may influence myofilament activation. Indirectly, calcium flux and myofilament activation are affected by the flow of other ions that comprise the cardiomyocyte action potential and channels that are under the control of the molecular clock. *Bmal1* knockout mice exhibited increased susceptibility to arrhythmias through disruption of the SCN5A sodium channel (Schroder et al. 2013), while circadian variations in potassium channel-interacting protein 2 (KChIP2) altered cellular repolarization and shortened action potential duration (Fotiadis and Forger 2013). While not directly affecting myofilament function or composition, these circadian variations in action potential characteristics may impact the ability of the sarcomere to generate force.

In summary, the fundamental contribution of cardiac myofilaments to myocardial performance positions them to have a substantial impact on heart function. Despite the central role for the sarcomeric proteins in setting cardiac performance, there are many unexplored questions about the effects of circadian rhythms on the myofilaments. There is intriguing evidence to support the idea of circadian regulation in processes specific to sarcomeric signaling and remodeling. This new area presents an exciting opportunity to explore an unknown but potentially valuable area of circadian regulation of cardiac physiology.

8.6 Therapeutic Applications

Translational chronocardiology applies circadian rhythms biology to clinical cardiology to improve diagnosis and treatment of CVD. Time-of-day specific disease markers (chronobiomarkers) can be applied toward diagnosis and/or prognosis of CVD. Administering drug therapies at an optimal time-of-day (chronotherapy) can increase therapeutic efficacy while decreasing side effects (chronotherapy in hypertension is further discussed in Chap. 12). These applications are described in further detail below and are summarized in Fig. 8.2.

8.6.1 Definition of Biomarkers and Application of Diurnal Biomarkers

The standardized biomarker definition by National Institutes of Health is "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a*

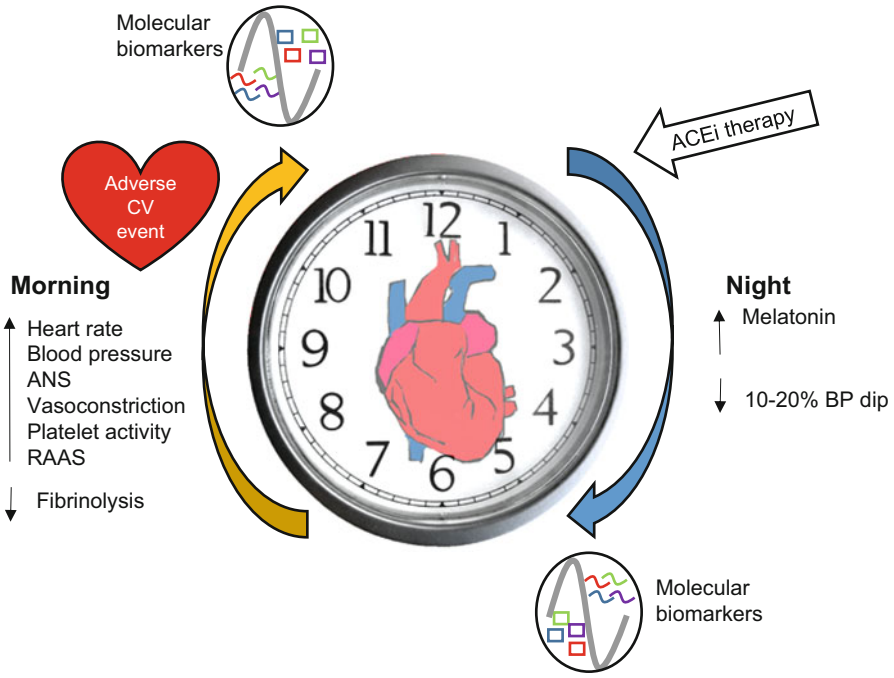

Fig. 8.2 Translational applications. The circadian mechanism is involved in the regulation of cardiovascular physiology, which can be used to the advantage of biomarkers and therapy. Different processes organized in time act on the cardiovascular system day vs. night. Molecular diurnal biomarkers—genes and proteins—can be advantageous as markers for diagnosis and prognosis of heart disease. Chronotherapy targets diurnal cycling of cardiovascular physiology, for example, angiotensin-converting enzyme inhibitor (ACEi) treatment in the evening is most effective at restoring nocturnal blood pressure and also for benefiting cardiac growth, renewal, and remodeling

therapeutic intervention" (Biomarkers Definitions Working 2001). Commonly accepted biomarker standards include profiling factors (e.g., diet, exercise, and smoking) for early prediction of CVD risk and molecular markers for the detection of myocardial necrosis, ischemia, inflammation, and/or hemodynamic stress commonly associated with CVD [as reviewed by Maisel et al. (2006) and Jaffe et al. (2006)]. The chronomic approach to biomarker discovery goes one step further, aiming to identify rhythmic biomarker expression over 24 h day/night cycles that differ between health and disease. This can lead to identifying de novo biomarkers of CVD. It is worth noting that traditional biomarkers are discovered in the light period based on nocturnal rodent studies (i.e., when scientists are working but rodents are in their sleep phase) and then extrapolated to diurnal human physiology. In contrast, chronobiomarkers are biomarker candidates discovered across the entire diurnal cycle for application to human disease.

8.6.2 Identifying Biomarkers by High-Throughput Technology

Microarray studies have provided a novel approach for determining body time in target tissues. This was demonstrated as proof of concept by Ueda et al., in which body time was determined based on expression of ~100 genes across a molecular timetable (Ueda et al. 2004). Measuring internal body time was subsequently demonstrated by blood metabolomics approaches in mice (Minami et al. 2009) and in humans (Kasukawa et al. 2012). These can also be applied clinically; we demonstrated time-of-day chronobiomarkers of murine cardiac hypertrophy from microarray gene expression profiling and a novel algorithm termed DeltaGene (Tsimakouridze et al. 2012).

8.6.3 Identifying Biomarkers Using Proteomics Approaches

Proteins are key biological mediators and rate-limiting factors directly involved in cellular processes important for health and disease. They also represent an ideal target for de novo identification of chronobiomarkers. This has been demonstrated as a proof-of-concept study in mice, using blood plasma samples collected across the 24 h day/night cycle and analyzed by surface-enhanced laser desorption and ionization (SELDI) mass spectrometry (Martino et al. 2007b). For clinical translation, blood would be the most advantageous tissue to collect from, as it does not require invasive organ sampling. For example, low nocturnal serum melatonin levels in patients with STEMI are predictive of a second adverse cardiovascular event, demonstrating the value of proteomic approaches (Dominguez-Rodriguez et al. 2006). It is worth noting that standardized sampling time implemented for population-based studies, in addition to generating new biomarkers, can also help to ensure consistency and reduce variability in existing markers (Rudnicka et al. 2007).

8.6.4 Identifying Diurnal Biomarkers Using Physiologic Approaches

Diurnal BP rhythms can also be considered a physiologic chronobiomarker of health and disease. That is, most people have a nocturnal dip in BP of ~10 % as compared to the day time (Millar-Craig et al. 1978). However, hypertensive non-dippers (patients who do not experience the anticipated drop in BP at night) are at an increased risk of cardiovascular disease (Verdecchia et al. 1993). Moreover, patients with elevated nocturnal BP have an increased incidence of adverse cardiovascular events compared to those with normal BP profiles (Ohkubo

et al. 2002). Importantly, several international societies including the International Society for Chronobiology (ISC), American Association of Medical Chronobiology and Chronotherapeutics (AAMCC), Spanish Society of Applied Chronobiology, Chronotherapy, and Vascular Risk (SECAC), Spanish Society of Atherosclerosis (SEA), and Romanian Society of Internal Medicine (RSIM) have recommended ambulatory 24 h BP monitoring as a superior technique for diagnosing and assessing risk of CVD (Hermida et al. 2013).

8.6.5 Chronotherapy Benefits Hypertension and Non-dippers

Chronotherapy aims to deliver the ideal drug dosage at the most efficacious time by considering the body's time-of-day rhythms (Smolensky and Haus 2001). The beneficial effects of chronotherapy in hypertension are perhaps best studied to date [as reviewed by Smolensky et al. (2010)]. Low-dose aspirin is a more effective antihypertensive agent when administered to patients at bedtime as compared to waking, possibly relating to a slower clearance rate at that time (Hermida et al. 2003). Chronotherapy has also been proposed for other medications that regulate BP, including the calcium channel blockers verapamil and diltiazem, the beta-blocker propranolol, and angiotensin-converting enzyme inhibitors (ACEi) enalapril, quinapril, and ramipril [as reviewed by Prisant (2004), Guo and Stein (2003), and Hermida et al. (2005)]. For example, nighttime ramipril administration reduces BP in the wake phase just as daytime ramipril; however, only nighttime administration partially restores the nocturnal dipper profile in 115 hypertensive patients with 6 weeks of ramipril monotherapy (Hermida and Ayala 2009). Chronotherapeutic applications have also been demonstrated with angiotensin-receptor blockers (ARB), as bedtime administration of telmisartan more effectively restores nocturnal BP compared to wake time administration (Hermida et al. 2007). Dosing time also applies to combination therapies such as valsartan (an ARB) and amlodipine (a calcium channel blocker) which more effectively restore nocturnal BP when coadministered at bedtime compared to the day time [e.g., Hermida et al. (2010a)]. A key physiologic application of decreased nocturnal BP is to mimic the dipper profile associated with normal cardiovascular physiology. Indeed, the Ambulatory Blood Pressure Monitoring and Cardiovascular Events (MAPEC) clinical trial showed that patients administered antihypertensive medications at bedtime had decreased prevalence of non-dipping hypertension and at the 6-year follow-up they had fewer adverse cardiac events, vs. those who took their medications upon awakening (Hermida et al. 2010b). Hermida and colleagues further developed this topic in Chap. 12.

8.6.6 Chronotherapy Benefits Cardiac Remodeling

Chronotherapy can also directly benefit cardiac remodeling (structure and function) in heart disease. Experimentally, sleep-time administration of the short-acting ACEi captopril significantly improves cardiac function and reduces hypertrophy and fibrosis, as compared to wake-time ACEi, in the pressure overload cardiac hypertrophy model in mice (Martino et al. 2011). The sleep-time benefits of ACEi captopril chronotherapy correlate with the diurnal gene expression profiles of renin-angiotensin-aldosterone system (RAAS). That is, timing ACEi treatments with the peak cycling of the RAAS pathway can be more effective and benefit the heart health of patients. In terms of future directions, since ACEi are commonly prescribed post-MI, it would be interesting to determine whether chronotherapy also benefits cardiac remodeling in this clinically relevant disease. Additional future directions include (1) beta-blockers for time-of-day administration; (2) antihypertensive drug combinations, such studies are currently limited (Hermida et al. 2010a, 2011); and (3) approaches for compliance at specific times of day or night, such as specialized drug delivery systems [as reviewed by Smolensky and Peppas (2007)]. In summary, chronotherapy is a promising new approach for benefiting patients clinically.

8.7 Conclusion

The circadian clock mechanism is crucial to the cardiovascular system. It plays a role in regulating diurnal HR, BP, ANS, and cardiac metabolism, all of which underlie healthy cardiovascular physiology. It is important for underlying rhythms in cardiac gene expression. Mechanistically, regulation of both extrinsic (SCN) and intrinsic (heart) circadian regulated factors creates a time of greatest cardiac vulnerability and influence timing of onset of adverse cardiovascular events (e.g., MI, VT, and SCD). Circadian rhythms and their disturbance influence disease pathophysiology and outcome. Moreover, investigation of circadian rhythms in the cardiovascular system has revealed new roles in regulating sarcomere function and ultimately cardiac contractility in health and disease. They also lead to new diagnostic and therapeutic opportunities for patients with CVD.

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Chapter 9
Regulation of Immunity by the Circadian Clock

Alba de Juan, David Druzd, Louise Ince, and Christoph Scheiermann

Abstract Over the last few years, molecular evidence has clearly shown a direct impact of daily rhythms on the immune system, both in steady state and under inflammatory conditions. Circadian oscillations have been demonstrated in the regulation of cytokine and chemokine levels as well as immune cell numbers in blood and tissues. A possible explanation for these rhythms is that species are more prone to be exposed to a variety of acute threats such as injuries and microbial infections at specific times during their circadian cycle. Also some chronic diseases follow a circadian rhythm, indicating that a greater understanding of the underlying molecular mechanisms will permit the development of new and improved therapeutic strategies for treating inflammatory diseases that incorporate the element of time.

Keywords Circadian immunity • Leukocyte recruitment • Molecular clock • Inflammatory mediators • Endogenous oscillations

9.1 Introduction

The concept of circadian rhythms was introduced in the late 1950s to characterize self-sustained rhythms that occur under constant conditions (Halberg et al. 1959). These rhythms exhibit a period length of about 24 h and play a critical role in the adaptation of organisms to their environment (Golombek and Rosenstein 2010). Increasing evidence suggests that molecular links exist between circadian rhythms and the constituents of the immune system (Arjona et al. 2012; Curtis et al. 2014; Druzd et al. 2014; Lange et al. 2010; Scheiermann et al. 2013; Labrecque and Cermakian 2015). The data indicate that cyclical expression of pro-inflammatory factors in immune cells and recruitment of these cells to tissues are regulated by the molecular clock, which can affect disease outcome. In this chapter, we will

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illustrate the impact of oscillations in immune parameters on the immune system under both physiological and pathological conditions.

9.2 Circadian Clock

As a result of complex molecular interrelations, organisms are able to adjust their body clock optimally to the environmental conditions to which they are exposed. Light, as the main environmental entrainment factor or *Zeitgeber* ("time giver"), operates as a master synchronizer that orchestrates the behavioral rest–activity cycle. Light is processed in the eye via photosensitive retinal ganglion cells (pRGCs) (Lucas et al. 1999), which are non-image forming cells that transfer photic input to the suprachiasmatic nuclei (SCN) via the retinohypothalamic tract. Situated above the optic chiasm in the anterior hypothalamus, the SCN consist of approximately 20,000 highly interconnected neurons. This is critical for the rapid establishment of phase coherence once a light stimulus is detected (Reppert and Weaver 2001).

At the molecular level, the clock consists of multiple sets of transcription factors that drive robust autoregulatory transcription–translation feedback loops (TTFLs). Photic input synchronizes the transcription of clock genes, which consist of the positive regulators *Bmal1* (brain and muscle Arnt-like protein 1, encoded by *Arntl*), *Clock* (circadian locomotor output cycles kaput), and *Rorα* (RAR-related orphan receptor) as well as the negative regulators cryptochrome (*Cry1–2*), period (*Per1–3*), *Rev-erbα*, and *Rev-erbβ* (encoded by *Nr1d1* and *Nr1d2*, respectively) (Levi and Schibler 2007). Heterodimerization of BMAL1/CLOCK in the cytoplasm forms a transcription factor complex, which binds to Enhancer Box (E-Box)-sequences in the promoters of both positive and negative regulators, resulting in an autoregulatory feedback loop, which is reset by a new light cycle. BMAL1/CLOCK also induce expression of clock-controlled genes that then drive circadian output processes. For detailed information on the molecular mechanisms of the clock, the reader is encouraged to consult Chap. 1.

9.3 Clock Components in the Regulation of the Immune System

There are numerous examples of direct interactions between the molecular clock and the immune system. Clock proteins can have anti- or pro-inflammatory properties as demonstrated by the respective immune phenotypes of mice deficient in molecular clock components (Table 9.1).

Table 9.1 Immune phenotypes of molecular clock mutant mice

Genotype	Phenotype	References
Anti-inflammatory clock genes		
Bmal1$^{-/-}$ (*Arntl*)	No oscillations in CFU-Cs, CXCR4, or *Cxcl12*	Lucas et al. (2008), Mendez-Ferrer et al. (2008)
	Reduced levels of mature B cells	Sun et al. (2006)
LysM-Cre × *Bmal1*$^{flox/flox}$	Loss of circadian IL-6 expression after LPS stimulation	Gibbs et al. (2012)
	Increased serum levels of IL-1β, IL-6, IFNγ, and CCL2; enhanced lethality to *Listeria monocytogenes*; higher macrophage numbers in white adipose tissue	Nguyen et al. (2013)
Cry1$^{-/-}$	Enhanced levels of IL-6	Narasimamurthy et al. (2012)
Cry1$^{-/-}$ *Cry2*$^{-/-}$	Increased joint swelling in an arthritis model	Hashiramoto et al. (2010)
	Increased NF-κB activation; enhanced levels of IL-6, TNFα, *Cxcl1* and *iNos*; heightened sensitivity to LPS	Narasimamurthy et al. (2012)
Rorα$^{-/-}$	Defective T and B cell development; enhanced IL-6 and TNFα production in mast cells after LPS stimulation	Dzhagalov et al. (2004)
Rorα$^{sg/sg}$	Enhanced sensitivity to LPS	Stapleton et al. (2005)
Nr1d1$^{-/-}$ (*Rev-erbα*)	Loss of circadian IL-6 expression after LPS stimulation	Gibbs et al. (2012)
Pro-inflammatory clock genes		
Clock$^{-/-}$	Reduced nuclear accumulation of p65 and NF-κB activation	Spengler et al. (2012)
Clock$^{\Delta 19}$	Reduced responsiveness to LPS in mouse embryonic fibroblasts	Bellet et al. (2012)
	Reduced responsiveness to LPS in bone marrow-derived macrophages; reduced gut colonization of *Salmonella enterica*	Bellet et al. (2013)
Per1$^{-/-}$	Altered rhythms of IFNγ, perforin, and granzyme B in splenic NK cells	Logan et al. (2013)
Per2$^{-/-}$	No rhythm and decreased expression of IFNγ and IL-1β in spleen	Liu et al. (2006)
	Decreased IFNγ and IL-1β levels in serum; increased resistance to LPS-induced endotoxic shock	Arjona and Sarkar (2006)
Nr1d1$^{-/-}$ (*Rev-erbα*)	Promotion of T_H17 cell differentiation	Yu et al. (2013)

CFU-Cs colony-forming units in culture, *CXCR4* CXC chemokine receptor 4, *CXCL* chemokine (C-X-C motif) ligand, *IL* interleukin, *IFNγ* interferon gamma, *TNFα* tumor necrosis factor alpha; *NF-κB* nuclear factor κB, *iNOS* inducible nitric oxide synthase, *LPS* lipopolysaccharide, T_H17 T helper 17 cells

9.3.1 Anti-inflammatory Clock Genes

BMAL1 BMAL1 is a central component of the molecular clock and the only single gene whose deficiency renders the organism's behavior immediately arrhythmic (Bunger et al. 2000). Also in the immune system BMAL1 appears to play a central role in the regulation of inflammatory reactions. *Bmal1*$^{-/-}$ mice exhibit a diverse phenotype ranging from calcified joints to accelerated aging (Bunger et al. 2005; Kondratov et al. 2006). Investigators have, therefore, generated animal models using conditional targeting strategies to define the immune phenotype caused by a lineage-specific deletion of BMAL1. Myeloid cell-specific deletion experiments of BMAL1 using *LysM-Cre* × *Bmal1*$^{flox/flox}$ mice identified peritoneal macrophages as key to a rhythmic response to systemic administration of lipopolysaccharides (LPS). In isolated myeloid cells from BMAL1-deficient mice as well as in the whole animals, IL-6 responses to LPS were higher during the resting phase than in wild-type (WT) mice and displayed no oscillations (Gibbs et al. 2012). Using the same genetic tools, Nguyen et al. demonstrated a direct suppressive effect of BMAL1 on the inflammatory response of myeloid cells. Under steady-state conditions, BMAL1 was shown to rhythmically bind to the promoter regions of *Ccl2*, *Ccl8*, and *S100a8*, genes that encode pro-inflammatory chemokines (Nguyen et al. 2013). BMAL1 recruits members of the polycomb repressor complex (PRC2) to these genes, which results in epigenetic silencing of the loci. When BMAL1 is deleted in the myeloid lineage this inhibitory feedback is ablated, resulting in increased systemic cytokine expression. This is of relevance for disease as survival of myeloid *Bmal1*$^{-/-}$ mice that were infected with the bacterium *Listeria monocytogenes* was dramatically reduced compared to control animals (Nguyen et al. 2013). Thus, rhythmic repression of inflammatory genes by BMAL1 and its associated epigenetic silencing complex is critical to appropriately balance inflammatory reactions.

CRY Proteins Fibroblasts isolated from *Cry1*$^{-/-}$ but not *Cry2*$^{-/-}$ mice showed enhanced levels of IL-6. *Cry1* and *Cry2* double knockout (*Cry1*$^{-/-}$*Cry2*$^{-/-}$) fibroblasts and mice, however, exhibited a dramatically enhanced increase in the expression of the pro-inflammatory molecules IL-6, TNF-α, and iNOS compared to WT animals and a heightened sensitivity to LPS (Narasimamurthy et al. 2012). This effect appeared to be due to the loss of CRY binding to and suppression of adenylyl cyclase, resulting in enhanced levels of cyclic AMP (cAMP) and constitutive protein kinase A activity, with enhanced expression of the pro-inflammatory nuclear factor κB (NF-κB) complex as consequence. In a model of arthritis, induced with a mixture of anti-type II collagen-antibody and LPS, *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice were shown to exhibit enhanced joint swelling and a higher inflammatory cytokine profile (Hashiramoto et al. 2010). These results support the notion that lack of both *Cry* genes results in a synergistic activation of pro-inflammatory cytokines, indicating a role in the regulation of inflammatory cytokine expression.

RORα *Rorα*$^{-/-}$ mice exhibit dramatic reductions in cell numbers in spleen and thymus compared to littermate controls, which is likely caused by a defective lymphocyte microenvironment (Dzhagalov et al. 2004). In addition, production of IFNγ in *Rorα*-deficient CD8 T cells was enhanced upon activation as was TNF-α and IL-6 release in mast cells and macrophages. Similarly, mice exhibiting the neurological mutation staggerer (sg) (*RORα*$^{sg/sg}$) displayed an exacerbation of lung inflammation after LPS instillation (Stapleton et al. 2005). These data indicate *RORα* as an important negative modulator of the immune system.

REV-ERBα *Rev-erba*$^{-/-}$ animals exhibit reduced frequencies of pro-inflammatory T_H17 cells in the gut (discussed below) (Yu et al. 2013). On the other hand, *Rev-erba*$^{-/-}$ mice showed enhanced levels of IL-6 at the onset of the resting phase after stimulation with LPS compared to WT animals and no oscillations in the cytokine (Gibbs et al. 2012). In addition, administration of the synthetic REV-ERBα agonist GSK4112 suppressed IL-6 release from macrophages while knocking down the gene enhanced *Il6* expression (Gibbs et al. 2012). The same effect was also seen for transcription of *Ccl2* (Sato et al. 2014). Mechanistically, REV-ERBα was shown to directly bind to a *Rev-erba* motif in the *Ccl2* promoter region and suppress transcription. These data indicate that REV-ERBα can display pro- and anti-inflammatory properties.

9.3.2 Pro-inflammatory Clock Genes

CLOCK Bone marrow-derived macrophages (BMDM) from *Clock*$^{\Delta 19}$ mutant mice display a reduced expression of the pro-inflammatory cytokines *Il6*, *Il1b*, *Tnfa*, *Ifnb*, *Cxcl1*, and *Ccl2* after incubation with LPS and reduced secretion of IL-6 and TNF-α (Bellet et al. 2013). After infection with the bacterium *Salmonella typhimurium* animals also exhibited lower and nonrhythmic bacterial content in colon and Peyer's patches. CLOCK is a histone acetyl transferase (HAT) and can potently acetylate and activate the subunit p65 of the key pro-inflammatory complex NF-κB (Doi et al. 2006). As a consequence of CLOCK deficiency, activation of NF-κB in response to inflammatory stimuli in mouse embryonic fibroblasts and primary hepatocytes harvested from *Clock*$^{-/-}$ mice was significantly reduced compared to WT cells (Spengler et al. 2012). In contrast, *Clock*$^{\Delta 19}$ mutants, which showed reduced transactivation capacity of CLOCK on E-box-containing promoters exhibited no defect in the ability of CLOCK to upregulate NF-κB-dependent promoters (Spengler et al. 2012). These results indicate two distinct functions of CLOCK, as a modulator of the transcriptional activation of NF-κB-responsive elements and as a transactivator in complex with BMAL1 on E-box-responsive promoters.

PER Proteins *Per2*$^{-/-}$ mice exhibit a decrease in the expression of IFNγ and IL-1β in serum, resulting in a higher resistance of these animals to lipopolysaccharide (LPS)-induced systemic inflammation and lethality (Arjona and Sarkar 2006; Liu et

al. 2006). Similarly, *Per1*$^{-/-}$ mice display significantly altered rhythms of IFNγ and the cytolytic factors perforin and granzyme B in splenic natural killer (NK) cells (Logan et al. 2013). Together, these data provide strong evidence for a broad impact of multiple clock components on immune homeostasis and direct molecular regulation of pro-inflammatory factors.

9.4 Oscillations in Immunomodulatory Parameters

Chemokines and cytokines are key humoral parameters of the immune system and exhibit fluctuations in a daily manner with peaks and troughs at specific Zeitgeber times (ZT, time after an environmental cue such as light) (Table 9.2). IL-1β, IL-6, and TNFα, cytokines that are released during the acute phase response to inflammation, exhibit peak values in serum or peritoneal fluid around the onset of the behavioral activity phase (Arjona and Sarkar 2005; Gibbs et al. 2012; Nguyen et al. 2013; Young et al. 1995). Also, cell surface receptors involved in the innate immune response and the recognition of pathogens oscillate. Macrophages possess an autonomous circadian clock—an intrinsic oscillation in their gene expression pattern over 24 h (Gibbs et al. 2012; Keller et al. 2009). Toll-like receptor (TLR) 9, a pathogen receptor that is critical for the recognition of bacterial DNA motifs, was shown to oscillate in splenic macrophages and B lymphocytes, peaking during the activity phase of mice (Silver et al. 2012). In contrast, mRNA expression of the TLR family members *Tlr4* and *Tlr7*, important for the detection of LPS and single-stranded RNA, respectively, did not show substantial daily fluctuations. However, TLR4-associated receptors such as CD14 and downstream signaling components were shown to oscillate (Keller et al. 2009). This suggests that fluctuations exist in whole signaling cascades involved in pathogen recognition, supporting the notion that these mechanisms have developed to counter microbial encounter at specific times.

9.5 Rhythms in Leukocyte Trafficking

Besides these fluctuations in immune-modulatory molecules, other cellular components oscillate in the organism. Leukocyte counts vary dramatically in blood with lymphocytes, neutrophils, monocytes, and eosinophils exhibiting a peak during the day in rodents and during the night in humans, both during their respective resting phases. In contrast to leukocyte counts, platelets and red blood cell numbers do not fluctuate (Scheiermann et al. 2012).

Lymphocytes T lymphocyte populations in human blood were shown to be differentially regulated via release of cortisol and catecholamines (Dimitrov et al. 2009). Within the CD4 and CD8 T cell subsets, naive T cells displayed pronounced

Table 9.2 Rhythms in pro-inflammatory molecules

Class	Molecule	Species	Source	Acrophase	Trough	References
Cytokines	IL-1β	Mouse	Peritoneal fluid	ZT8	ZT12	Nguyen et al. (2013)
	IL-2	Human	Serum	1 pm	1 am	Young et al. (1995)
	IL-5	Mouse	Serum	10 am	10 pm	Nussbaum et al. (2013)
	IL-6	Mouse	Serum	CT12	CT0	Gibbs et al. (2012)
	IL-10	Human	Serum	7:30 pm	2 am	Young et al. (1995)
	IL-12	Mouse	Serum	CT12	CT0	Gibbs et al. (2012)
	TNFα	Human	Serum	1:30 pm	1:30 am	Young et al. (1995)
		Rat	NK cells	ZT14–24	ZT2–6	Arjona and Sarkar (2005)
	IFNγ	Rat	NK cells	ZT14–24	ZT2–6	Arjona and Sarkar (2005)
	GM-CSF	Human	Serum	7:30 pm	3 am	Young et al. (1995)
Chemokines	CCL2	Mouse	Muscle endothelial cells	ZT13	ZT1	Scheiermann et al. (2012)
			Serum	CT12	CT0	Gibbs et al. (2012)
	CCL5		Serum	CT12	CT0	Gibbs et al. (2012)
	CXCL1		Serum	CT12	CT0	Gibbs et al. (2012)
	CXCL12		BM microenvironment	ZT21	ZT9	Mendez-Ferrer et al. (2008)
Chemokine receptors	CX_3CR1	Human	CD8 T cells	9 am	9 pm	Dimitrov et al. (2009)
	CXCR4	Mouse	Neutrophils	ZT17	ZT5	Casanova-Acebes et al. (2013)
			HSCs	ZT13	ZT5	Lucas et al. (2008)
		Human	T cells	9 am	9 pm	Dimitrov et al. (2009)
Adhesion molecules	E-selectin	Mouse	BM endothelial cells	ZT13	ZT5	Scheiermann et al. (2012)
	P-selectin		BM endothelial cells	ZT13	ZT5	Scheiermann et al. (2012)
	L-selectin		Neutrophils	ZT5	ZT17	Casanova-Acebes et al. (2013)
	ICAM1		Muscle endothelial cells	ZT13	ZT5	Scheiermann et al. (2012)
	VCAM1		BM endothelial cells	ZT13	ZT5	Scheiermann et al. (2012)

(continued)

Table 9.2 (continued)

Class	Molecule	Species	Source	Acrophase	Trough	References
Others	Granzyme B	Rat	NK cells	ZT14–24	ZT2–6	Arjona and Sarkar (2005)
	Perforin		NK cells	ZT14–24	ZT2–6	Arjona and Sarkar (2005)
	TLR9	Mouse	Splenic B cells, macrophages	ZT19	ZT7	Silver et al. (2012)

IL interleukin, *IFNγ* interferon gamma, *TNFα* tumor necrosis factor alpha, *CCL* chemokine (C-C motif) ligand, *CXCL* chemokine (C-X-C motif) ligand, *CX_3CR* CX_3C chemokine receptor, *CXCR4* CXC chemokine receptor 4, *GM-CSF* granulocyte macrophage colony-stimulating factor, *HSC* hematopoietic stem cell, *ICAM1* intercellular adhesion molecule 1, *VCAM1* vascular cell adhesion molecule 1, *GM-CSF* granulocyte macrophage colony-stimulating factor, *TLR9* toll-like receptor 9, *NK* natural killer, *ZT* Zeitgeber time (time after the onset of light, with lights on at ZT0 and ZT24 and off at ZT12), *CT* circadian time (time under constant conditions in a nonrhythmic environment).

circadian rhythms with a daytime nadir. Naive T cells were negatively correlated with cortisol rhythms, decreased after low-dose cortisol infusion, and showed highest expression of CXCR4. Effector CD8 T cells in contrast were positively correlated with epinephrine rhythms, increased after epinephrine infusion, and showed highest expression of adrenergic receptors and CX3 chemokine receptor 1 (CX_3CR1, also known as fractalkine receptor) (Dimitrov et al. 2009). Recent data also implicate circadian clocks in the maturation process of Interleukin-17-producing $CD4^+$ T helper (T_H17) cells, pro-inflammatory immune cells that protect against bacterial and fungal infections (Yu et al. 2013). The development of T_H17 is regulated by the orphan nuclear receptor RORγt (Ivanov et al. 2006). The transcription factor NFIL3 (nuclear factor, interleukin 3 regulated; also known as E4BP4) binds to the *Rorγt* promoter and represses expression. NFIL3 itself is repressed by the core clock component REV-ERBα, which is induced by CLOCK/BMAL1. Thus, expression of REV-ERBα promotes T_H17 cell development by indirectly activating RORγt, linking it to the circadian clock. Light-cycle disruption increased intestinal T_H17 cell frequencies indicating that lineage specification of a key adaptive immune cell is under circadian control (Yu et al. 2013).

Neutrophils Neutrophils exhibit the shortest life span among leukocytes, lasting only several hours in the circulation. This circulation period is associated with an aging process illustrated by altered expression levels of two adhesion molecules on the neutrophil surface, L-selectin (also known as CD62L) and the chemokine receptor CXCR4. As neutrophils age, they lose L-selectin and enhance their levels of CXCR4, thus turning into $CD62L^{LO}$ $CXCR4^{HI}$ cells (Martin et al. 2003). This process represents an activation phenotype in vitro and has also been observed in vivo (discussed in more detail below) (Casanova-Acebes et al. 2013; Martin et al. 2003).

Monocytes Mouse monocytes are categorized as being either inflammatory or resident based on their migration to various tissues and the expression of surface molecules such as Ly6C, chemokine receptor 2 (CCR2), and CX_3CR1 (Geissmann et al. 2010). Nguyen et al. showed that the inflammatory, but not the resident, monocyte subset exhibits a diurnal oscillation that drives rhythmic migratory behavior. Using *LysM-Cre* × $Bmal1^{flox/flox}$ mice to deplete the circadian gene *Bmal1* in the myeloid lineage, the authors made the observation that the oscillations ceased to exist (Nguyen et al. 2013). This suggests that intrinsic rhythms within myeloid cells can orchestrate the trafficking behavior of inflammatory monocytes and thus can regulate their own numbers in the organism.

Eosinophils Eosinophil numbers in blood are regulated by serum IL-5 levels, which have recently been shown to be maintained by long-lived type 2 innate lymphoid cells (ILC2s) (Nussbaum et al. 2013). ILC2s are found in peripheral tissues such as intestine, lung, and skin but are absent from lymphoid tissues. Importantly, circulating IL-5 and eosinophil levels could be modulated by caloric input and were shown to be dramatically reduced after 16 h of fasting. This was dependent on the neuropeptide vasoactive intestinal peptide (VIP), which is highly

expressed in intestinal neurons. VIP was shown to signal via VIP receptor type (VPAC) 2 on ILC2 cells (Nussbaum et al. 2013). Thus, ILC2s in the small intestine increase the IL-5 production after nutrient intake, which indicates how eosinophil numbers can be controlled in a circadian manner. These data may thus provide a mechanism for the phenotype seen in mice deficient in VPAC2 that exhibit a delayed infiltration of eosinophils after allergic challenge.

The oscillating leukocyte count in blood is the result of rhythmic release of cells from the bone marrow as well as an oscillatory recruitment to tissues. Blood cellular components are produced in the bone marrow, in a process termed hematopoiesis. From the bone marrow, hematopoietic stem and progenitor cells (HSPCs) as well as mature leukocyte populations are disseminated into the circulation. HSCPs are mobilized into blood at the beginning of the rest phase (Mendez-Ferrer et al. 2008). This mobilization can be enforced by granulocyte-colony stimulating factor (G-CSF) and is controlled locally by sympathetic nerves. Mobilization occurs in antiphase with the expression of the chemokine CXCL12 (also known as stromal cell-derived factor-1), the main retention factor for HSPCs in the bone marrow (Aiuti et al. 1997). The circadian regulation of HSPC release from the bone marrow suggests a role for the central clock in the orchestration of the expression of numerous target genes in peripheral tissues (Levi and Schibler 2007; Liu et al. 2007). Indeed, circulating colony-forming units in culture (CFU-C) counts and *Cxcl12* mRNA expression levels were not found to oscillate in *Bmal1*$^{-/-}$ mice (Mendez-Ferrer et al. 2008). In addition to CXCL12, its receptor on HSPCs, CXCR4, is under circadian control, indicating an optimal efficiency of interactions due to peak expression of the binding partners at similar times (Lucas et al. 2008).

Homeostasis of hematopoietic stem cells in their bone marrow niche appears to be dependent on the clearance of neutrophils from the circulation under steady-state conditions. Aged CD62L^{LO} CXCR4^{HI} neutrophils are eliminated from blood at the end of the resting period in mice (Casanova-Acebes et al. 2013). These neutrophils infiltrate the bone marrow and promote reductions in the size and function of the hematopoietic stem cell niche. CAR (CXCL12-abundant reticular) niche cell numbers in the murine bone marrow oscillate over the course of a day exhibiting a peak before the onset of the activity phase (ZT9) (Casanova-Acebes et al. 2013). Rhythmic modulation of the niche depends on macrophages and activation of cholesterol-sensing nuclear liver X receptors (LXR), which stimulate the hematopoietic stem cell niche to mobilize HSPCs into blood. The transcript levels of two LXR target genes, *Abca1* and *Mertk*, involved in cholesterol efflux and phagocytic uptake, respectively, exhibited fluctuations in antiphase with those of circulating neutrophils, which is of relevance as mice deficient in LXRα and β showed an impaired mobilization of HSPCs (Casanova-Acebes et al. 2013). These data demonstrate that the phagocytosis of aged neutrophils from blood by macrophages acts as a rheostat for the release of new hematopoietic cells into the circulation.

Leukocyte trafficking across different tissues occurs in a circadian manner, coordinated by the expression of cell adhesion molecules and chemokines. Circadian rhythms in leukocyte recruitment have been described in bone marrow,

regulated at the steps within the leukocyte adhesion cascade of rolling and adhesion due to expression of the homing receptors P-selectin, E-selectin, and VCAM1 (Scheiermann et al. 2012). Instead, in the cremaster muscle rhythms were apparent at the level of leukocyte adhesion and extravasation, downstream steps in the leukocyte adhesion cascade, which was mediated by rhythmic expression of endothelial cell ICAM-1 and CCL2 (Scheiermann et al. 2012). The circadian differences in leukocyte recruitment in these tissues indicate substantial diurnal changes in the ability of endothelial cells to recruit leukocytes. These data suggest that oscillations in tissue- and leukocyte-specific expression of key promigratory factors determine the nature of oscillatory leukocyte infiltration.

9.6 Adrenergic Control

The sympathetic nervous system (SNS) is an important synchronizer of circadian rhythms in peripheral tissues. Signals from the brain are transmitted via the SNS to peripheral tissues by catecholamines through adrenergic receptors (Elenkov et al. 2000b). Levels of locally released norepinephrine (NE), as well as circulating epinephrine, which is produced by the adrenal gland, exhibit peak levels at the onset of the activity phase (Maestroni et al. 1998). Over the past 20 years multiple studies have implicated catecholamines as important neuromodulators of immunity in lymphoid organs. In contrast to the classical doctrine of neurophysiology, in which the synapse is the primary site of neuronal information transmission (Tansey 1997), there are several areas, such as blood vessels and lymphoid organs, where nerves terminate without direct synaptic contact with target cells (Vizi 1984). As a result of stimulation by NE released from nerves and humoral epinephrine, leukocyte migration, proliferation, and function are affected in a diverse manner. NE and epinephrine mediate their effects on target cells via stimulation of two receptor classes: alpha (α_{1-2}) and beta (β_{1-3}) adrenergic receptors. The most prevalent of these is the β_2-adrenergic receptor, which is expressed by virtually all leukocyte subsets (Elenkov et al. 2000a), with the important exception of Th2 cells (Sanders et al. 1997). Leukocyte subsets differ in their β_2-adrenergic receptor density, with natural killer (NK) cells exhibiting the greatest and helper T cells the lowest number of receptors. More recent data implicate an important role for epinephrine in the modulation of invariant natural killer T (iNKT) cell function in the liver as blocking sympathetic signals was protective in mice in a stroke model (Wong et al. 2011). α-adrenergic receptors (1 and 2 subtypes) are mostly found on alveolar and peritoneal macrophages and can be expressed on other immune cells under certain pathologic conditions (Heijnen et al. 1996). These data suggest key roles for an oscillatory sympathetic tone on immune cell function. For an overview, the reader is referred to an in-depth review on this topic (Elenkov et al. 2000b).

A previously unrecognized role of the SNS is played in the bone marrow hematopoietic stem cell niche. Nestin$^+$ cells are key components of the niche due to their expression of high levels of CXCL12 as well as other niche factors

(Kunisaki et al. 2013). Nestin^{+} cells are associated with sympathetic nerves alongside arteries and express the β_3-adrenergic receptor on their surface, which sympathetic nerves target to modulate niche cell activity locally. Adrenergic signals lead to degradation of the transcription factor SP1 and rapid downregulation of bone marrow CXCL12 levels (Mendez-Ferrer et al. 2008).

Adrenergic nerves can also modulate interactions between hematopoietic cells and endothelial cells required for the recruitment process into tissues. Endothelial cells in both bone marrow and skeletal muscle express β_2- and β_3-adrenergic receptors (Steinle et al. 2003). Neural adrenergic input modulates the expression of adhesion receptors in the microvasculature and induces a higher expression during the active phase with functional implications for leukocyte trafficking: *Adrβ2*$^{-/-}$ and *Adrβ3*$^{-/-}$ animals deficient in either β_2- or β_3-adrenergic receptors show no more oscillatory recruitment to bone marrow or muscle tissue (Scheiermann et al. 2012). Furthermore, administration of β_2- or β_3-adrenergic receptor agonists (clenbuterol or BRL37344, respectively) was found to greatly enhance bone marrow engraftment in transplantation studies by upregulating adhesion molecules required for the homing process. These events are orchestrated by sympathetic adrenergic nerves, which modulate fluctuations in the chemokines and adhesion molecules on both hematopoietic and nonhematopoietic cells required for hematopoietic cell trafficking (Scheiermann et al. 2012).

9.7 Rhythms in Diseases of the Immune System

It has been described that certain diseases follow a circadian rhythm demonstrated by an aggravation of symptoms according to the time of the day. This is the case for various chronic inflammatory diseases such as rheumatoid arthritis (RA) whose symptoms—morning stiffness—display a significant peak at the onset of behavioral activity (Cutolo 2012; Straub and Cutolo 2007). This is associated with a rhythmic oscillation in serum levels of the pro-inflammatory cytokines IL-6 and TNFα. In RA patients, the peak level of TNFα and IL-6 has been reported to occur between 6:00 and 7:00 AM and remains elevated until noon, when levels in healthy subjects have already decreased (Straub and Cutolo 2007).

Cardiovascular events such as myocardial ischemia (Mulcahy et al. 1988; Parker et al. 1994) and infarction (Muller et al. 1985) also represent a higher risk during the morning in patients. Tofler et al. reported that this severity appears to be related with day–night alterations in adrenergic activity and associated systemic arterial pressure (Tofler et al. 1995). In addition to the increased occurrence of myocardial infarction in the morning, infarct sizes were also found to be larger, suggesting that rhythmic leukocyte infiltration may play a contributing role (Suarez-Barrientos et al. 2011). Thus, a variety of diseases exhibit striking rhythms in occurrence and symptom aggravation. Gaining better knowledge into the underlying molecular events will provide new insights into treatment options.

9.8 Conclusions

The brain and the immune system are the two major adaptive systems of the body. Under both steady-state conditions and during an immune response, both communicate extensively with each other to maintain homeostasis. The suprachiasmatic nuclei synchronize an intrinsic circadian oscillation described in many immune cells and tissues via neural and humoral means. This fluctuation is a vital regulator of leukocyte migratory behavior and function. Studies also indicate that leukocyte trafficking is regulated in a rhythmic manner at every stage of the maturation process. The regulation of the hematopoietic stem cell niche, the mobilization of hematopoietic cells into blood, the recruitment from the circulation into tissues, the expression of immunomodulatory factors, and their phagocytosis all occur in a rhythmic fashion. Over the last few years, new insights have provided molecular evidence for intricate interactions between circadian rhythms and the immune system. The fact of a rhythmic immune system should be kept in mind when considering developing new therapies to target inflammatory diseases.

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Chapter 10
Rhythms in the Digestive System

David B. Rhoads, Lynne L. Levitsky, and Ali Tavakkoli

Abstract This chapter reviews how circadian rhythms in the gastrointestinal (GI) tract are established and contribute to homeostasis. Circadian rhythms in the digestive system are shaped by a combination of endogenous rhythms and our activity/rest schedules. Most rhythms have obvious rationales, for instance, the temporal partitioning of opposing cellular functions including absorption vs. proliferation and glucose disposal vs. glucose production. Circadian clocks in peripheral tissues are cell-autonomous molecular mechanisms that maintain 24-h periodicity and drive cyclic expression of many rhythmic functions or clock outputs. In general, GI clocks coordinate the induction of physiological processes to match temporal needs. The feeding rhythm is a potent entrainment signal, presumed to operate by shifting the phases of GI clocks and, consequently, clock outputs. Disturbance of clock operation by mutation or detrimental environmental stimuli can disrupt proper functioning of the digestive system and could lead to adverse metabolic changes, obesity, and diabetes. A more thorough understanding of intestinal clocks and their role in nutrition and homeostasis should enhance our ability to address health issues of modern life.

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10.1 Chapter Overview

We are all more or less aware of many daily rhythms associated with the digestive system, such as appetite (hunger/satiety), thirst, specific food cravings, borborygmus, and regularity (or irregularity) of bowel movements. However, we may not be aware of numerous additional 24-h cycles that are nonetheless important for gastrointestinal (GI) absorption, secretion, metabolism, and proliferation. This chapter will explore the major circadian rhythms of the GI tract; their maintenance and coordination with the central circadian clock; and their role in homeostasis. We hope that the reader will develop a deeper appreciation of the extent of gut rhythms, their roles in health, and their underlying molecular and physiological bases.

Circadian rhythmicity in the digestive system is a prime example of evolutionary adaptation to the earth's 24-h day–night oscillation. Survival is dependent on acquiring food while avoiding predation. Efficiently balancing these often conflicting goals entails optimizing vigilance during one phase (i.e., day or night) to search for food and then hiding during the other phase when vigilance is compromised. This adaptation conserves energy by relying on simpler sense organs (adapted *only* to light *or* dark) while minimizing energy expenditures during rest. Circadian rhythmicity is particularly important in continuously renewing tissues such as the GI tract where expending biosynthetic energy near the end of a cell's life might be wasteful. Furthermore, consolidating nutrient intake leads to major metabolic shifts daily—from catabolism and utilization of nutrients to production of glucose and anabolic precursors—in maintaining homeostasis. In other words, circadian cycles in gene expression and enzyme activities enable temporal segregation of competing metabolic pathways. Importantly, many rhythms are *anticipatory*, so that processes such as absorption or hepatic glucose output begin to increase in sufficient time to attain adequate rates when needed. As such, many genes increase expression before they are needed each day and continue to do so for days or more in the absence of external stimuli, including light and feeding.

Circadian cycles in gene expression arise as outputs from circadian clocks present in most cells. As detailed in Chap. 1, an organism's circadian rhythmicity is generated by the central clock in the suprachiasmatic nuclei (SCN), consisting of interlocking positive and negative transcriptional loops. The SCN clock is free-running in that no stimulus is required to oscillate but its phase can be entrained by external stimuli, with light being the most powerful zeitgeber ("time-giver"). Circadian clocks are also present in most peripheral tissues (Balsalobre et al. 1998) and the GI tract is no exception (Sladek et al. 2007b; Froy and Chapnik 2007; Hoogerwerf et al. 2007, 2008; Pan and Hussain 2009; LeSauter et al. 2009; Balakrishnan et al. 2010a). Peripheral clocks in each organ system have their own phases (or sets of phases), generally delayed up to ~6 h from the central clock

(Panda et al. 2002; Kowalska and Brown 2007). The cycling transcription factors in the circadian clock also produce cyclic transcription of "non-clock" target genes, i.e., the clock-controlled genes (CCGs) (Lowrey and Takahashi 2004). For instance, rhythmic vasopressin release into the cerebrospinal fluid by the SCN (Schwartz and Reppert 1985) relies on transcriptional activation of the vasopressin gene by CLOCK:BMAL1 clock elements (Jin et al. 1999). DBP (D-box binding protein), a second early CCG example, is expressed in several tissues including liver that rhythmically enhances expression of albumin and other genes (Ripperger et al. 2000). As a transcription factor itself, DBP targets can thus be formally considered "second-order" clock output genes. It is important to distinguish between circadian rhythms driven by cyclic systemic signals, such as glucocorticoid-responsive genes, and the clock output genes described above, which arise cell-autonomously from endogenous circadian clock activity (Kornmann et al. 2007a).

The subset of cycling genes in a given tissue (0.5–9 %) is relatively specific, but each tissue subset shows remarkably little overlap (~10 %) between unrelated tissues (Duffield 2003). In addition to rhythmic transcription, other mechanisms such as mRNA stability, regulation by noncoding RNAs, translational efficiency, and posttranslational modification [reviewed in Kojima and Green (2014)] as well as epigenetic regulation (Eckel-Mahan and Sassone-Corsi 2013; Everett and Lazar 2014) substantially contribute to cyclic gene expression.

This chapter will focus on the GI tract proper, briefly delving into GI-associated organs and the brain as needed to understand GI rhythmicity.

10.2 Physiological Rhythms

Modern investigations into GI rhythms began in the 1970s, with studies focusing on rhythms in feeding/activity, intestinal function, and mucosal proliferation. Diurnal humans tend to be active in the day, whereas most rodent species employed for experimentation are nocturnal, i.e., active at night.

10.2.1 Feeding Rhythm

Nocturnal rodents feed predominantly at night (Zigmond et al. 1969; Kimura et al. 1970). Removal of the light:dark oscillation by providing constant light gave rise to the notion of cuing by photoperiod [see Polidarova et al. (2011)]. However, most rodent studies now employ constant dark (or dim light), a preferable constant condition due to masking by light, which suppresses rodent activity (Morin 2013). In constant dark, rodents develop a feeding rhythm with nearly 24-h periodicity (Nishida et al. 1978).

Scheer et al. (2013) examined the notion that appetite in humans is suppressed in the biological morning despite the overnight fast (breakfast is typically the smallest meal). In their study of 12 healthy adults, hunger for food in general as well as for all food classes was highest in the evening, when the largest meal is eaten. They speculated that the larger evening meal prepares one for an extended fast, whereas a smaller breakfast adapts one to the extended fast.

10.2.2 Proliferation Rhythms

Proliferation of mucosal cells in the rat GI tract exhibits circadian rhythmicity and generally peaks, as measured by ^{3}H-thymidine incorporation, at lights-on when rats cease activity (Scheving et al. 1978). In this particular study, the duodenum exhibited little rhythmicity, a pattern ascribed to the complexity of its proliferative compartment. Following intraperitoneal injection of epidermal growth factor (EGF) in mice, proliferation in the GI tract occurred sequentially with a cranial-caudal gradient (Scheving et al. 1979, 1980). Corresponding rhythms were also observed in villus height and cell number, with temporal phases that could be shifted by restricted feeding (RF) during the daytime (Stevenson et al. 1979). Expression of mRNA for *Wee1*, a cell cycle checkpoint gene, exhibits circadian rhythmicity in the mouse duodenum and colon, with several hours of phase delay in the latter tissue (Polidarova et al. 2009). In humans, DNA labeling of serial biopsies from oral and rectal mucosae revealed that cell replication was generally higher at night and lower in the afternoon, approximately opposite to that observed in nocturnal rodents (Marra et al. 1994). Circadian rhythmicity of the antiproliferative microRNA mir-16 may contribute to replication cycles (Balakrishnan et al. 2010b). The temporal segregation of the energy-requiring replication process from the activity/feeding phase, when demand for mucosal cell function is lower, has been judged beneficial (Scheving 2000).

10.2.3 Functional Rhythms

The earliest investigators to document rhythmicity in intestinal absorptive capacity in rats were Fuyura and Yagari with L-histidine (Furuya and Yugari 1971, 1974) and Fisher and Gardner with glucose (Fisher and Gardner 1976). These investigators concluded that the rhythm in uptake capacity was induced by the food consumption rhythm rather than by light per se. Three main points arose from these and further studies. First, with ad libitum feeding, light suppresses both feeding rhythm and sucrose rhythmicity, while scheduled feeding in either light or dark can entrain sucrose rhythmicity (Nishida et al. 1978). Second, that luminal exposure to nutrients is not required to establish circadian rhythmicity in intestinal functions was shown in two ways. Maltase and sucrase activities in isolated jejunal sacs of rats fed

ad libitum were rhythmic with the same circadian phase as the intact intestine despite the lack of luminal exposure to ingested nutrients (Saito et al. 1978). Similarly, discontinuous delivery of total parental nutrition led to rhythmic expression of several intestinal functions, including monosaccharide uptake, hydrolytic activities, and villus hyperplasia (Stevenson et al. 1980). Rhythmicity disappeared with continuous delivery. Finally, the increase in maltase and sucrase activities *before* the onset of eating indicated that expression rhythms are *anticipatory*, entrained by the previous intake rhythm (Saito et al. 1976). Thus, nutrient influx is a potent zeitgeber for the digestive system.

Entrainment of intestinal rhythms exhibits some plasticity. Rats can be entrained to twice-daily rhythmicity in hydrolase activities by dividing food availability into two 3-h bouts separated by 12 h (Furuya et al. 1979). However, twice-daily rhythmicity may be the limit because SCN-lesioned rats provided food divided into three daily bouts could still only entrain to at most two daily rounds of food anticipatory activity (FAA) (Stephan 1989). Furthermore, tissue circadian clocks appear limited to 24-h rhythmicity. Using rats carrying a *Per1-luc* transgene (Yamazaki et al. 2000), Davidson et al. (2003) examined the effect of twice-daily food entrainment on peripheral clocks in *Per1-luc* transgenic rats. Following adaptation to two 2-h bouts of food per day, one each in the middle of the night or day, FAA as assessed by wheel-running exhibited robust 12-h rhythmicity by 2.5 weeks. However, peripheral clocks as reported by Luciferase activity in explants from several GI tissues (esophagus, stomach, liver, and colon) retained 24-h rhythmicity with a phase consistent with entrainment to the nighttime feeding. These studies raise several questions regarding second antiphase peaks in intestinal activity or FAA. For instance, responses could arise from twice-daily systemic factors (non-cell-autonomous) or clock rhythms in tissues not assessed for *Per1-luc* transgene rhythmicity. More detailed studies are clearly needed to elucidate the mechanism(s) underlying twice-daily entrainment.

In the first studies at the molecular level, the Na^+/glucose cotransporter SGLT1 and the facilitated glucose transporters GLUT2 and GLUT5 were shown to exhibit circadian rhythmicity in both mRNA and protein (Corpe and Burant 1996). Transcriptional rhythms were subsequently shown to contribute to the expression rhythms of SGLT1, GLUT2, and sucrase (Rhoads et al. 1998). SGLT1 mRNA and protein rhythms persist in isolated, jejunal Thiry-Vella loops (Stearns et al. 2009), indicating that luminal exposure is not required as Saito et al. observed for sucrase activity (Saito et al. 1978). The H^+/peptide cotransporter PEPT1 also exhibits circadian rhythmicity (Pan et al. 2002). Of interest, these investigators showed that neither PEPT1 nor SGLT1 was rhythmically expressed in the kidney, as would be expected for its round-the-clock absorptive function. Inui's group further showed that DBP enhancers in the distal *Pept1* promoter (-0.78 and -0.63 kb) accounted for the rhythmicity of intestinal PEPT1 expression (Saito et al. 2008). These investigators concluded that PEPT1 rhythmicity was driven by this CCG rather than core clock components such as CLOCK/BMAL1 (see Chap. 1), as found for the Na^+/H^+ electrolyte exchanger NHE3 (Saifur Rohman et al. 2005) (see also Sect. 10.3.3). On the other hand, rhythmic binding of Bmal1 to

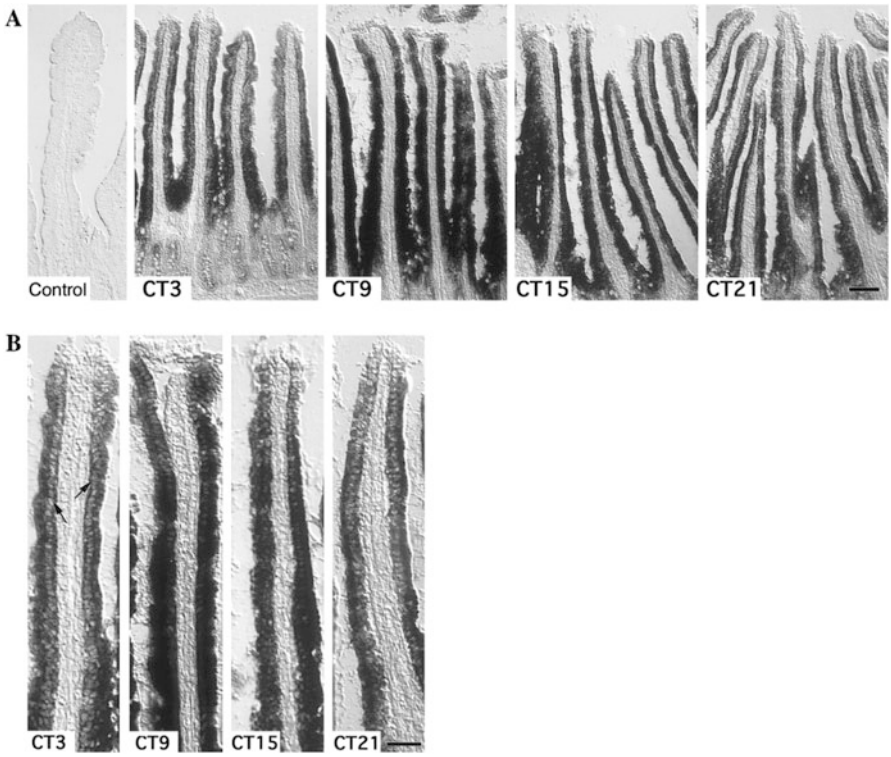

Fig. 10.1 In situ hybridization labeling of SGLT1 mRNA in enterocytes of rat proximal small intestine. (**a**) The control picture was taken from tissue treated with the sense probe, whereas others were treated with the antisense probe. Details of digoxigenin–riboprobe synthesis and tissue preparation can be found in the original article. Pictures were taken with identical settings to allow comparison of labeling intensities. The labeling intensity of enterocytes from CT9 and CT15 was distinctly greater than that of enterocytes from the other two times. *Bar*, 100 μm. (**b**) Higher magnification view of single villi shown in (**a**). These villi were cut approximately in the vertical plane and show only a single layer of enterocytes. As in (**a**), the labeling intensities of enterocytes in villi from CT9 and CT15 were stronger than the other two, indicating higher expression levels of SGLT1 mRNA. *Bar*, 50 μm (Tavakkolizadeh et al. 2001)

the promoters of the genes for SGLT1, GLUT2, and GLUT5 as their expression increased provides evidence for the involvement of core clock components in their rhythmicity (Iwashina et al. 2011). The basis for nonrhythmic expression of hexose transporters and PEPT1 in kidney is currently unknown. Possibly, tissue-specific transcriptional repressors prevent activity of circadian enhancers.

Expression topography also exhibits periodicity (Tavakkolizadeh et al. 2001). For instance, SGLT1 mRNA exhibits a rhythmic expansion in abundance along the crypt–villus axis (Fig. 10.1). Enterocytes lower in the villus appear to transcribe SGLT1 mRNA continuously, while those toward the tip do so with circadian periodicity. This pattern suggests that "younger" cells are dedicated to increasing the expression of SGLT1 protein, while "older" cells merely replace turned-over

protein, but do so in phase with anticipated absorptive needs. The mechanism underlying this apparent differential rhythmicity as enterocytes migrate up the villus is currently unknown.

10.3 Circadian Clocks and Gene Expression Rhythms

Circadian clocks have been found in essentially all regions of the digestive system. Phases depend largely on the temporal pattern of food consumption and, as expected, rhythmic output gene phases (CCGs) exhibit a similar dependence. The best-characterized transcriptome is that in the liver (Damiola et al. 2000; Rey et al. 2011; Schupp et al. 2013; Masri et al. 2014; Du et al. 2014). The liver is covered in more detail in Chap. 5, but a few words are justified here due to the liver's role as the gateway for metabolic intake. First, the liver is very sensitive to feeding periodicity and so has provided much detail on the responses of the liver clock and CCGs to restricted and shifted feeding protocols. Second, its dramatic shifts in metabolism offer a fruitful system to elucidate the mechanisms of temporal pathway partitioning. For instance, the nuclear hormone receptor REV-ERBα is a core clock gene that orchestrates a circadian repression–derepression rhythm of many hepatic genes involved in lipid metabolism (Everett and Lazar 2014). Along with its redundant homolog REV-ERBβ, REV-ERBα recruits constitutively expressed nuclear corepressor 1 (NCor1) and histone deacetylase 3 (HDAC3) to target genes, thereby rhythmically repressing them. In contrast, the retinoic acid-related orphan receptor (ROR)-α, which binds to the same promoter elements, activates transcription in antiphase. Another example is the differential roles played by SIRT1 and SIRT6, two members of the sirtuin family of NAD^+-dependent deacetylases that independently interact with the CLOCK:BMAL1 transcriptional complex, in circadian control of many metabolic pathways (Chang and Guarente 2014). Sassone-Corsi's group (Masri et al. 2014) provided evidence that SIRT1 principally regulates genes involved in lipid and carbohydrate metabolism, whereas SIRT6 targets peptide and cofactor gene pathways. Third, culture techniques have been developed for long-term maintenance of differentiated, primary hepatocytes in vitro (Dunn et al. 1989). This technique was employed to show that hepatocytes isolated from PER2::Luciferase mice (Yoo et al. 2004) can maintain rhythmic expression of this circadian reporter for several weeks (Guenthner et al. 2014). While only weak local coupling was observed among individual hepatocyte clocks under the in vitro conditions used in this study, in contrast to the strong coupling observed for SCN neurons (Webb et al. 2009; Welsh et al. 2010), further studies may identify hepatic coupling factors that synchronize clocks in the liver (and other GI tissues). Fourth, the liver is readily transducible by viral gene delivery vectors (Herzog 2005). Moreover, the liver's size allows external detection of light from expressed Luciferase reporter genes in living, albeit hairless, mice (Saini et al. 2013). In the Saini et al. study, rhythmic light output from either a PER2::Luciferase or BMAL1::Luciferase transgene paralleled endogenous liver clock

expression under ad libitum and RF regimens. Most interesting, the more rapid phase shift of the liver clock to RF in SCN-lesioned mice vs. SCN-intact mice led these investigators to posit that the central clock "brakes" such shifts by producing signals, such as glucocorticoids (Le Minh et al. 2001), that can counteract but not overcome metabolic cues. Lastly (but certainly not finally), Schibler's group has generated a mouse line in which the liver clock (hepatocyte clocks, specifically) is inoperative unless doxycycline is added to the drinking water (Kornmann et al. 2007b). Interestingly, despite stoppage of the clock by suppression of *Bmal1* expression via REV-ERBα overexpression, *Per2* expression remained robustly circadian. Loss of *Per2* rhythmicity in tissue explants led these investigators to suggest that *Per2* was responding to cyclic systemic factors rather than exhibiting cell-autonomous oscillation.

With that introduction, we turn to other tissues of the digestive system.

10.3.1 Stomach

A circadian clock rhythm (PER1 and PER2) was detected in gastric oxyntic cells, which secrete the orexigenic hormone ghrelin in anticipation of meals (LeSauter et al. 2009). FAA is initiated by ghrelin injection in sated mice, but is blunted in $GHSR^{-/-}$ mice, which lack the ghrelin receptor (growth hormone secretagogue receptor). PER1/PER2 rhythms and ghrelin secretion were phase-shifted in parallel by RF. Ghrelin receptors are present on vagus afferents as well as the subfornical organ (SFO), which projects to hypothalamic appetite centers such as the arcuate nuclei, paraventricular nuclei, supraoptic nuclei, and lateral hypothalamus. From these data, the authors proposed that oxyntic cells are components of the food-entrainable oscillator (FEO; see below). This conclusion is supported by the finding that $GHSR^{-/-}$ mice also take longer to entrain to RF under dark:dark conditions (Lamont et al. 2014). It should be noted that rodents generally exhibit a single nightly ghrelin peak (Bertani et al. 2010), whereas humans often exhibit several daily peaks corresponding to the individual meal pattern (Frecka and Mattes 2008). Humans can also exhibit a fourth ghrelin peak at night, interpreted as a "rebound" peak approximately 5 h after the third daytime meal (Spiegel et al. 2011).

10.3.2 Small Intestine

Circadian clock genes are present in the jejunum (Froy and Chapnik 2007; Balakrishnan et al. 2010a). In Froy and Chapnik's study, total mouse jejunum and the isolated crypt cell compartment exhibited mRNA rhythms in several clock genes (*Bmal1*, *Per1*, *Per2*, and *Cry1*) as well as for several Toll-like receptors (TLRs; *Tlr2*, *Tlr3*, *Tlr4*, and *Tlr5*), pattern recognition receptors involved in host

immunity (Cario 2005). However, it was not determined whether the TLR rhythms were driven by the clock, feeding rhythm, or other factors.

The thorough analysis of intestinal clocks performed by Pan et al. provided many details (Pan and Hussain 2009). Importantly, expression was assessed with mRNA, protein, and activity in most cases. First, circadian clock gene mRNAs and protein (*Clock*, *Bmal1*, *Per1-3*, and *Cry1-2*) were expressed throughout the mouse small intestine and colon, including the epithelial cell compartment. In the jejunum, all clock genes (except *Clock*) exhibited circadian rhythmicity. Second, clock gene rhythms phase-shifted in response to RF for 2 h during the light phase, a pattern that was maintained after transfer to constant dark for 5 days. In contrast, loss of rhythms after transfer to constant light for 5 days, despite the RF regimen, demonstrated the strong suppressive effect of light on operation of the circadian clock. Third, in dominant negative $Clock^{mt/mt}$ mice [$Clock^{\Delta 19/\Delta 19}$ of Vitaterna et al. (1994)], rhythms of circadian clock genes were lost or dampened and were not regained even with RF. Fourth, expression rhythms of absorptive genes (SGLT1, GLUT2, GLUT5, and PepT1) were phase-shifted by RF in wild-type mice but absent in $Clock^{mt/mt}$ mice regardless of feeding regimen. Fifth, lipid absorption (as measured by radiolabeled cholesterol and triolein) exhibited circadian rhythmicity in wild-type mice but was chronically elevated in $Clock^{mt/mt}$ mice. These effects were observed both in isolated intestinal loops and isolated enterocytes, thereby removing gastric acid secretion and systemic hormones as confounding factors. The investigators maintained that increased lipid absorption was etiologic in the obesity and hyperlipidemia observed in $Clock^{mt/mt}$ mice (Turek et al. 2005). Sixth, circadian rhythmicity of numerous lipid metabolic genes with expression responsive to light and/or feeding regimen was lost or attenuated in $Clock^{mt/mt}$ mice. Ultimately, the authors proposed that *Clock* is necessary for food entrainment of the intestine, but that more definitive conclusions would require tissue-specific knockouts and examining mutations other than the dominant negative $Clock^{\Delta 19/\Delta 19}$.

10.3.3 Colon

The colon has several functions, including absorption of water and electrolytes, propulsion, storage, and defecation, most of which exhibit circadian rhythmicity. The first systematic studies of circadian clock genes in the GI tract proper were performed in this tissue. In one (Hoogerwerf et al. 2007), cycling of *Bmal1*, *Per* genes, and *Cry* genes was observed in colonic crypts and the myenteric plexus of both the stomach and colon. Restricted daytime feeding reversed the clock phases in the GI tract but not in the SCN. Moreover, truncal vagotomy had no effect on the peripheral clocks, despite the radical nature of this procedure. Hoogerwerf's group later performed transcriptional profiling on total distal colon and found cycling in <4 % of transcripts (Hoogerwerf et al. 2008). Notably, over half of the transcripts were in processes consistent with a continuously renewing compartment, e.g.,

cellular growth and proliferation; DNA replication, recombination, and repair; cell development; cell cycle; and cell death.

The other study focused on the colonic epithelium (Sladek et al. 2007b), which examined expression of the Na^+/H^+ electrolyte exchanger NHE3, earlier shown to be a renal CCG induced by CLOCK:BMAL1 dimers (Saifur Rohman et al. 2005). Sladek et al. demonstrated circadian expression of NHE3 along with clock gene mRNAs (*Per1*, *Per2*, *Cry1*, *Bmal1*, *Clock*, and *Rev-erbα*) and proteins (PER1 and BMAL1). The colonic clock phase paralleled that in the liver. Phase advance of the NHE3 rhythm with RF led the investigators to suggest that the colonic clock drove its expression rhythm to match need.

Earlier, expression of circadian clock genes (*Per1*, *Per2* and *Clock* mRNAs; PER1 and CLOCK proteins) was detected in human colonic crypts from human biopsies, but, due to single-time sampling, cycling could not be assessed (Pardini et al. 2005).

10.3.4 Pancreas

Circadian clocks have been found in both the exocrine pancreas (Damiola et al. 2000; Muhlbauer et al. 2004) and pancreatic islets (Marcheva et al. 2010; Sadacca et al. 2011). Particularly germane to the digestive system, a functional clock in pancreatic β cells is necessary for proper function and proliferation (Marcheva et al. 2010). Global ablation of either *Clock* or *Bmal1* impairs glucose-stimulated insulin secretion in an age-dependent manner, ascribed in part to partial compensation by the liver in these total knockout lines (Marcheva et al. 2010). As such, pancreas-specific *Bmal1* knockout [via Pdx-Cre (Gu et al. 2002)] leads to earlier defects in glucose homeostasis. *Rev-erbα* is also rhythmically expressed in both pancreatic β cells and α cells and is necessary for glucose-regulated secretion of insulin or glucagon, respectively (Vieira et al. 2012, 2013). Further treatment of rhythms in the endocrine system can be found in Chap. 2.

10.3.5 Ontogeny

In the rat colon, rhythmicity of *Per1*, *Per2*, *Cry1*, *Bmal1*, and *Rev-erbα* (but not *Clock*) was detected on embryonic day 20 (E20) (Polidarova et al. 2014). However, all mRNA expression peaks clustered near subjective (maternal) lights-off and each exhibited a unique pattern of phase-shift or periodicity loss during development through postnatal day 30 (P30), the last point tested. The complexity of the observed patterns may reflect tissue differentiation and growth kinetics combined with changes in nutrient intake. For core clock genes in the liver, only *Rev-erbα* exhibited rhythmic expression on E19, but essentially all clock genes examined

developed the adult pattern of rhythmicity by P30 (Sladek et al. 2007a). [The rat SCN clock develops rhythmicity between E19 and P10 (Shearman et al. 1997; Sumova et al. 2008; Christ et al. 2012).] The lack of rhythmicity in intestinal sucrase and maltase activities in E19 or E20 rat fetuses is consistent with these data (Stevenson et al. 1977).

10.4 Coordination of Rhythms

In this section, the interactions of the GI tract with the central clock and other peripheral systems are reviewed. A consistent observation is that RF during the daytime in rodents can dissociate digestive system clocks from the SCN clock, which continues to follow the light cycle (Damiola et al. 2000). Such phase-shifted rhythms are entrained as they persist for several days after transfer to constant conditions and/or food deprivation.

10.4.1 Feeding Rhythms

The periodicity of eating is a complex process [for reviews, see Saper and Fuller (2007), Blum et al. (2012), and Menaker et al. (2013)]. Experimental paradigms including various light conditions (light:dark, light:light, or dark:dark) and feeding regimens (ad libitum vs. RF) have revealed many insights on how the digestive system responds to food intake patterns. Humans typically fall between ad libitum and RF by exercising free will within a consolidated sleep schedule. It is broadly accepted that GI clocks follow the feeding rhythm regardless of how that rhythm is established. In any case, an established feeding rhythm is characterized by several features: (1) clock phase; (2) functional rhythms, many of which precede or *anticipate* the onset of eating; (3) behavioral changes, e.g., wheel-running, that usually also *anticipate* the onset of eating; and (4) physiological changes, e.g., hormone secretion and temperature increase.

Events surrounding a feeding rhythm are coordinated by the interaction of multiple neural and peripheral sites. Evidence has been provided for input from several hypothalamic regions, e.g., the arcuate nuclei, paraventricular nuclei, supraoptic nuclei, and the dorsomedial, lateral, and ventromedial hypothalamus [for instance, see Stephan (2002), Gooley et al. (2006), Stenvers et al. (2012), Cagampang and Bruce (2012), Tahara and Shibata (2013), and Patton and Mistlberger (2013)]. Rhythmic secretion of neurohormones such as vasopressin, vasoactive intestinal polypeptide, prokineticin-2, orexins, pituitary adenylate cyclase-activating peptide, and transforming growth factor-α [reviewed in Cagampang and Bruce (2012)] and ghrelin (Cowley et al. 2003; Cabral et al. 2013) as well as systemic hormones such as leptin (Kalsbeek et al. 2010), insulin (Diaz-Munoz et al. 2000), glucocorticoids (Chung et al. 2011), and ghrelin

(LeSauter et al. 2009; Spiegel et al. 2011) also contribute. The jury is still out on the involvement of the enteric nervous system, but, as noted above, these neurons do express clocks that entrain with RF (Hoogerwerf et al. 2007). Lastly, the vagus has been implicated in transmitting signals on nutrient status directly to the brain from the small intestine (Lee et al. 2012; de Lartigue et al. 2014) as well as the hepatic portal vein (Mithieux 2014).

10.4.2 Hunger and Satiety

Several brain areas are involved in assessing energy status. The subfornical organ, a circumventricular organ in the lamina terminalis protruding into the third ventricle, has received attention because the absence of the blood–brain barrier gives its neurons access to plasma constituents (Smith and Ferguson 2010). Neurons have been identified that respond to glucose (Medeiros et al. 2012), insulin (Lakhi et al. 2013), and ghrelin (Pulman et al. 2006) among others. This pathway gives the central nervous system direct access to the energy status of peripheral tissues, thereby providing a possible mechanism to entrain an appetite rhythm.

Another recent approach has focused on the detection of glucose in the cerebrospinal fluid, which is lower but proportional to the plasma glucose concentration. Central to this sensing pathway is signaling from tanycytes, modified ependymal cells lining the third ventricle in contact with the cerebrospinal fluid, of which the β1-tanycyte subset sends projections to appetite-regulating neurons in the arcuate nucleus (Garcia et al. 2001; Langlet 2014). β1-tanycytes appear to be glucose-sensing cells (Cortes-Campos et al. 2011; Orellana et al. 2012) that express GLUT2 (Garcia et al. 2003) and glucokinase (GK) (Millan et al. 2010), both components of the glucose sensor shared by rodent hepatocytes and pancreatic β cells (Thorens 2001). [Parenthetically, human β cells use GLUT1 rather than GLUT2 (De Vos et al. 1995).] Both hepatocytes and β1-tanycytes also express the glucokinase regulatory protein (GKRP) that effects the glucose-mediated GK translocation between the nucleus and cytoplasm to regulate its activity (Agius 2008). However, tanycytes translocate GK to the nucleus in response to high glucose (Salgado et al. 2014) opposite to the direction observed in hepatocytes (Toyoda et al. 1997; Miwa et al. 1990). To test the notion that glucose metabolism by β1-tanycytes is involved in appetite, GLUT2 activity was inhibited by knockdown with short hairpin RNA (P Llanos, Magister Thesis, unpublished data). Of particular interest, knockdown of β1-tanycyte GLUT2 by intraventricular infusion of Ad-shGLUT2 induced a significant increase in food consumption and body weight gain compared with control infusion (Ad-shβGal). This result suggests that falling cerebrospinal glucose, a reflection of falling plasma glucose, activates brain centers involved in feeding rhythmicity and can thereby inform the central nervous system about both the current nutritional status and the recent timing of nutrient influx, thereby creating an anticipatory rhythm. In this context, translocation of β1-tanycyte GK to the nucleus under high glucose would flatten the glucose metabolic response,

thereby generating a more linear signal to glucose. In contrast, hepatocyte GK translocation to the cytoplasm by high glucose serves as a rectifier, regulating the direction of glucose flow according to nutritional status.

10.5 Rhythms in Health and Disease

In this last section, we touch on a few areas not covered above.

10.5.1 Coordination of the Cell Cycle with the Circadian Clock

Several studies have provided evidence that smooth operation of the circadian clock is necessary to curtail tumorigenesis [for reviews, see Gery and Koeffler (2010), Innominato et al. (2010), and Brown (2014)]. While the particular mechanistic pathways are yet unclear, a compelling notion is that the circadian clock may mediate critical decisions necessary for normal stem cell replication vs. asymmetric division (Brown 2014). Indeed, each stem cell population may have a unique relationship with its endogenous clock.

10.5.2 Parenteral Nutrition

Data on the effect of parenteral nutrition human digestive rhythms are relatively sparse [reviewed briefly by Stenvers et al. (2012)]. An earlier review (Matuchansky et al. 1992) described the development of cyclic parenteral nutrition (CPN). In general, one or all macronutrients are delivered nocturnally, largely for the convenience of recipients' activity schedule. Advantages of discontinuous glucose infusion included more normal rhythms for insulin/glucagon secretion and lipogenesis/lipolysis. One disadvantage is increased nocturnal urine production (Boncompain-Gerard et al. 2000). Essentially all studies aim to show that cyclic parenteral nutrition is as effective as continuous parenteral nutrition [see meta-analysis by Stout and Cober (2010)]. Thus, testing whether matching parenteral nutrition schedules to gut clocks would enhance the management of malabsorption conditions and/or patient well-being has not been systematically explored.

In rodents, total parenteral nutrition may be able to shift the SCN clock. As noted above, daytime RF dissociates the liver clocks from the SCN, which remains entrained to the light cycle (Damiola et al. 2000). In contrast, Miki et al. (2003) showed that diurnal provision of total parenteral nutrition for 7 days phase-shifted expression of *Per2* and *Dbp* mRNA abundances in both the liver and the SCN,

although SCN phases shifted only 8 h vs. 12 h for the liver. The authors hypothesized that total parenteral nutrition facilitated the SCN shift either because it provided a stronger nutritional signal than oral feeding or it eliminated the need to reverse the activity rhythm. It would be illuminating to examine other markers of feeding entrainment in this model, e.g., temperature and hormonal rhythms.

10.5.3 Stomach

Gastric acid secretion exhibits circadian rhythmicity (higher at night) (Moore and Englert 1970). However, because this rhythmicity is abolished by vagotomy (Moore 1991), parasympathetic innervation likely has greater influence than tissue clocks.

10.5.4 Intestinal Motility

Colonic motility is a complex process entailing both high- and low-amplitude local or propagating contractions (Bassotti et al. 1999). Rhythmicity of colonic motility is dependent on circadian clocks (Hoogerwerf 2010). Neuronal nitric oxide synthase (nNOS) produces nitric oxide (NO) that regulates motility in the colon as well as other regions of the GI tract (Takahashi 2003) and was found to be rhythmically expressed in the colon (Hoogerwerf et al. 2008). Arrhythmic double *Per1*/*Per2* knockout mice (Zheng et al. 2001) lost rhythmicity in several colonic functions such as fecal output and intracolonic pressure changes, presumably due to the loss of circadian signaling (Hoogerwerf et al. 2010). Loss of colonic rhythmicity in mice lacking a functional nNOS gene led these authors to suggest that nNOS is an essential component of the circadian mechanism controlling colonic rhythmicity. In humans, colonic motility is suppressed at night except, interestingly enough, during rapid-eye movement (REM) sleep (Furukawa et al. 1994), likely due to increased parasympathetic activity.

This chapter would be incomplete without mentioning the "circadian hormone" melatonin. A substantial amount of melatonin is produced by the gut (Bubenik 2008) estimated to contain several-hundred-fold more than the pineal gland (Huether et al. 1992). Gut microbiota might also provide a minor additional source (Hardeland and Poeggeler 2003). Exogenous melatonin affects both intestinal myenteric motor complexes (MMCs) in rats (Merle et al. 2000) and colonic motility in humans (Lu et al. 2005). Diurnal changes occur in motor activities and, to some extent, melatonin secretion, but only colonic motility has been directly linked to the circadian clock (Hoogerwerf 2010). However, food-anticipatory gastric and duodenal motility changes could be entrained in rats under RF that persisted for almost a week after feeding was delayed by 8 h (Comperatore and Stephan 1987). In humans, interdigestive MMCs occur predominantly at night (Scott et al. 2006) but

are driven by autonomic input (Husebye 1999) and tend to be interrupted by ingestion (Thompson et al. 1980; Deloose et al. 2012). Disruption of the MMC responses to feeding and hormonal stimulation by vagotomy in shrews provides evidence that MMCs are modulated by vagal input (Miyano et al. 2013). GI melatonin has paracrine function as well as being released into both the bloodstream (endocrine) and intestinal lumen ("exocrine") (Bubenik 2008). Release is relatively constant but is increased either by eating or food deprivation, an apparent paradox ascribed to melatonin's ability to slow intestinal transit time, thereby optimizing nutrient extraction at both extremes (Bubenik 2008). Ultimately, neither MMCs nor GI melatonin production has yet been linked to gut circadian clocks. Circumstantial evidence tends to favor derivative causes for any observed rhythmicity.

10.5.5 Sleep

Appetite is clearly linked to sleep, but inversely so. Unfortunately, the current trend toward less sleep (Czeisler 2013) has had adverse effects on health. Disrupted, short, or poor sleep increases appetite and adversely impacts glucose homeostasis and other metabolic parameters, disrupts several endocrine axes, and increases the risk of obesity and diabetes (Spiegel et al. 2009; Hanlon and Van Cauter 2011; Leproult and Van Cauter 2010). Moreover, prolonged sleep restriction combined with circadian disruption rapidly leads to adverse metabolic consequences (Buxton et al. 2012). In the Buxton et al.'s study, subjects replicated a "rotating shift" for 3 weeks of 28-h days with <6 h sleep per day as well as a delayed eating schedule. Decreases were observed in resting metabolic rate and meal-induced insulin secretion, the latter leading to longer postprandial glycemic excursions. Subjects recovered pre-study status within 9 days. The authors concluded that these metabolic impairments provide an explanation for the increased risk of obesity and diabetes with shift work. Sleep and the consequences of its disruption are covered further in Chaps. 3 and 5.

10.5.6 Chronotherapy

Cytotoxic therapies against tumors (e.g., chemotherapy and radiation) are effective because they target dividing cells, preferentially sparing most terminally differentiated cells. As such, the GI tract and other constantly renewing compartments are particularly sensitive, leading to side effects such as nausea and loss of appetite. Appropriate timing of treatments to increase tolerance to chemotherapy by administering cytotoxic therapies at the nadir of gut proliferation was first proposed in 1973 (Halberg et al. 1973). Theoretical aspects of timing cytotoxic treatments to minimize their impact on the GI tract were reviewed by Bjarnason and Jordan

(2002); similar arguments were made with regard to ionizing radiation (Haus 2002). Circadian rhythmicity in the expression of rat dihydropyrimidine dehydrogenase (DPD), the rate-limiting enzyme in 5-fluorouracil (5-FU) breakdown (Abolmaali et al. 2009), may explain this drug's greater tolerance when administered at night. TPN appears to reduce DPD expression in rats, possibly increasing 5-FU toxicity (Taniguchi et al. 2003), but sample timing was not noted in this study. A more detailed treatment of chronotherapy in cancer can be found in Chap. 11.

Several intestinal drug transporters also show circadian rhythmicity in expression (Stearns et al. 2008), potentially permitting lower dosages and/or increased efficacy with appropriate timing. For instance, pharmacokinetics of the oral antibiotic ceftibuten correspond to the expression rhythm of its transporter PEPT1 (Pan et al. 2003). Chronotherapy in the management of hypertension is covered in Chap. 12.

10.5.7 Intestinal Microbiota

We end by examining circadian aspects of our relationship with the microbial inhabitants in our gut. The gut microbiome has a substantial impact on health (Dethlefsen et al. 2007; Greenblum et al. 2012). The interaction between the microbiome and circadian functions in the ileum and distal colon was examined by Chambon's group (Mukherji et al. 2013). Mice in which gut microbiota were eliminated by antibiotics exhibited alterations in clock gene expression (largely amplitude rather than phase) but, more importantly, a degradation in metabolic parameters (hyperglycemia and hyperlipidemia) and a remarkable chronic overproduction of corticosterone. This last result was traced to ileal corticosterone production, ultimately caused by exaggerated JNK and IKKβ signaling due to the absence of their suppression by bacterial lipopolysaccharide (LPS). As such, delivery of LPS (as well as other manipulations) reversed both the hypercorticosteroidism and the metabolic parameters.

Thaiss et al. very recently documented circadian rhythmicity in the abundance of several taxa of intestinal microflora (Thaiss et al. 2014). Moreover, cycling was observed for approximately one-fourth of the bacterial pathways profiled, including such processes as metabolism of nucleotides, amino acids, and mucus. Rhythmicity of cycling taxa was lost in double *Per1*/*Per2* knockout mice (Zheng et al. 2001) but could be restored by RF. Fascinating results were obtained when mice were subjected to a "jet-lag" schedule, in which the day was alternately extended or shortened by 8 h every 3 days. Jet-lagged mice exhibited aberrant diurnal fluctuations in their microbiota, increased weight gain, and impaired glucose tolerance, all properties that could be transferred to germ-free mice by fecal transplantation. Most provocative was that fecal transplantation from jet-lagged humans (n=2) could confer similar metabolic impairments to germ-free mice. Clearly, this is an area that warrants much further study.

10.6 Summary and Future Directions

Circadian rhythms in the GI tract affect many areas of our daily behavior. However, the structural and functional diversity of GI tract and its associated tissues make overall study particularly challenging. While the digestive system exhibits flexibility to a variety of environmental conditions, mounting evidence indicates that there are limits. Clearly, departure from regular, adequate sleep and/or eating schedules significantly impact health. In addition, the extent to which population variation affects responses to departures from normative diurnal behavior is largely unexplored. Moreover, many studies rely on correlations to implicate circadian clocks in various cyclical processes. Defining how gut clocks function in establishing intestinal rhythms will require more mechanistic studies. Conversely, tracing the nutrient signaling pathways from the GI tract to neural centers regulating appetite rhythms may reveal targets useful in the treatment of obesity and diabetes. Ultimately, a deeper knowledge of gut circadian physiology is expected to help in addressing many modern health issues. By way of concluding, we offer some thoughts for directions that might enhance our understanding.

10.6.1 Gaps, Caveats, and Intriguing Approaches

- The liver is an excellent example for which the dependence of rhythmic functions on hepatocyte circadian clocks has been elegantly traced. However, in the GI tract, we are far from knowing which rhythmic functions are dependent on the endogenous clocks vs. systemic stimuli. Most conclusions are based on global clock gene knockouts, which may influence extra-GI tissues, or coordinate phase-shifts to RF, which do not prove causality. As such, tissue-specific and/or conditional knockouts of circadian genes in the GI tract proper would be useful in defining the linkages between the circadian clock and output gene expression. For instance, Villin-Cre (el Marjou et al. 2004) would be useful in targeting epithelial cells.
- Genome-wide association studies (GWAS) may reveal predictors of tolerability to shift work. Are some individuals genetically better suited to frequent time-shifts (Rosenberg et al. 2014)? Melatonin phasing appears to be one biomarker (Mirick and Davis 2008; Griefahn et al. 2002), but other clock gene polymorphisms may also contribute. Of interest in this regard, early chronotypes ("early birds") have phase-advanced circadian clocks in their oral mucosae compared with late chronotypes ("night owls") (Novakova et al. 2013). Double vasopressin V1a and V1b receptor knockout mice that immediately entrain to photic phase shifts (Yamaguchi et al. 2013) offer a provocative model.
- As much of our knowledge on circadian rhythms at the molecular level is derived from experiments on nocturnal rodents, care must be taken in extending findings to humans, for instance in correlating molecular changes with behavior

responses. As such, typical baseline conditions such as an overnight fast likely have different effects on rodents vs. humans. In this context, glucose tolerance varies diurnally, peaking at the onset of the activity period in both rats (Kalsbeek et al. 2003; la Fleur et al. 2001) and humans (Gibbs et al. 2013; Morgan et al. 2012). Also, somatotropin and melatonin are secreted largely at night in both humans and rodents while glucocorticoids are largely secreted during the activity phase.

- The enteric nervous system, a neural network with ~500 million neurons (Lake and Heuckeroth 2013), remains mysterious. Does the myenteric plexus have its own circadian synchronizing mechanism or is it subservient to the GI and/or the SCN clocks?
- Next Generation Sequencing and RNA-Seq, both more rapid and powerful than microarray, will allow more detailed characterization of regional differences in peripheral clocks and CCGs (Kojima and Green 2014). These technologies should facilitate characterization of distinct circadian behaviors in the various GI regions. In addition, NGS and RNA-Seq should facilitate increasing sampling resolution. For instance, 1-h sampling intervals revealed that a substantial number of liver transcripts cycled at shorter than 24-h rhythmicity (ultradian rhythms) (Hughes et al. 2009).
- The generation of intestinal tissue in vitro from human pluripotent stem cells (Watson et al. 2014) may provide a system for more focused studies on the interactions between human circadian genes and intestinal CCGs.

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Chapter 11
Chronotherapy of Blood Pressure Medications to Improve Management of Hypertension and Reduce Vascular Risk

Ramón C. Hermida, Diana E. Ayala, Michael H. Smolensky, and Francesco Portaluppi

Abstract Correlation between blood pressure (BP) and target organ damage, cardiovascular disease (CVD) risk, and long-term prognosis is greater for ambulatory BP monitoring (ABPM) than daytime in-clinic measurements. Additionally, consistent evidence from numerous studies substantiates that ABPM-determined asleep BP mean is an independent and stronger predictor of CVD risk than the awake or 24 h means. Hence, cost-effective adequate control of sleep-time BP is of marked clinical relevance. Ingestion time, according to circadian rhythms, of hypertension medications of six different classes and their combinations significantly impacts the beneficial and/or adverse effects of these drugs. For example, because the high-amplitude circadian rhythm of the renin-angiotensin-aldosterone system activates during nighttime sleep, bedtime versus morning ingestion of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers (ARB) better controls the asleep BP mean, with additional benefit, independent of medication terminal half-life, of converting the 24 h BP profile into more normal dipper patterning. The MAPEC Study, first prospective randomized treatment-time investigation testing the worthiness of bedtime chronotherapy with ≥ 1 conventional hypertension medications to specifically target attenuation of asleep BP, demonstrated, relative to conventional morning therapy, significantly better reduction of CVD risk: adjusted hazard ratio (HR) of total CVD events (HR = 0.39, 95 % CI [0.29–0.51]; $P < 0.001$) and major CVD events, i.e., CVD deaths, myocardial

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infarctions, and ischemic and hemorrhagic strokes (HR = 0.33 [0.19–0.55]; $P < 0.001$). CVD risk reduction was strongest when bedtime treatment included an ARB. The MAPEC Study documents that the asleep BP mean is the most significant prognostic marker of CVD and stroke morbidity and mortality. Moreover, the MAPEC study also substantiates attenuation of the asleep BP mean by a bedtime hypertension treatment strategy since the entire daily dose of ≥1 hypertension medications significantly reduces CVD risk, both in the general hypertension population and in patients of greater vulnerability and enhanced CVD risk, i.e., those diagnosed with chronic kidney disease, diabetes, and resistant hypertension.

Keywords Hypertension chronotherapy • Asleep blood pressure • Cardiovascular risk • Ambulatory blood pressure monitoring • MAPEC study • Hygia project • Diabetes • Chronic kidney disease • Resistant hypertension

11.1 Introduction

Blood pressure (BP) exhibits a mostly predictable daily variation that results from the interrelationship of many physiologic, neuroendocrine, and environmental factors: (1) rest–activity associated changes in behavior (including activity routine and level, meal timings and content, mental stress, and posture); (2) external day–night divergence in ambient temperature, humidity, and noise; and (4) endogenous circadian (~24 h) variation in neuroendocrine, endothelial, vasoactive peptide, and hemodynamic parameters [for example, plasma noradrenaline and adrenaline (autonomic nervous system, ANS), atrial natriuretic and calcitonin gene-related peptides, and renin, angiotensin, and aldosterone (renin-angiotensin-aldosterone system, RAAS)] (Table 11.1) (Fabbian et al. 2013; Hermida et al. 2007c; Portaluppi and Smolensky 2007; Portaluppi et al. 2012).

In most persons with normotension or uncomplicated essential hypertension, both systolic (SBP) and diastolic BP (DBP) are not static as might be expected based upon the concept of homeostasis, but variable in a predictable fashion during the 24 h; both decline to lowest levels during sleep, rise upon awakening, and elevate to highest values during (usually daytime) activity. The 24 h BP pattern typically exhibits two peaks during the daytime activity span, the first one ~3 h after awakening and the second one around 12 h after awakening, with a slight decline between them (Fabbian et al. 2013; Hermida et al. 2001, 2002a, c, 2007c). The fall of BP during sleep is commonly quantified by the sleep-time relative BP decline (percent decrease in mean BP during nighttime sleep relative to the mean BP during daytime activity). Although individuals are broadly and arbitrarily designated as dippers when the sleep-time relative SBP decline is ≥10 % or non-dippers when <10 %, it is more appropriate to apply a more descriptive categorization as extreme-dippers (decline ≥20 %), dippers (decline ≥10 %), non-dippers (decline <10 %), and risers (decline <0 %, asleep SBP mean > awake SBP mean) (Hermida et al. 2013j; Mancia et al. 2013).

Table 11.1 Contribution of circadian rhythms, cyclic environmental phenomena and other factors to blood pressure 24-h patterning in normotension and hypertension

Endogenous circadian rhythms
• ANS (vagal/sympathetic tone)
• RAAS: plasma renin, angiotensin and aldosterone
• Endothelin, atrial natriuretic, calcitonin gene-related, and other peptides
• Balance: vasodilating/vasoconstricting substances
• Left ventricular ejection fraction
• Stroke volume/cardiac output
• Peripheral vascular resistance
• Renal function/glomerular filtration
• Sodium and water diuresis
• Blood volume
Exogenous day–night cycles
• Noise level
• Ambient heat load
• Posture (upright daytime/supine asleep)
• Physical exertion/stress
• Cognitive load/mental stress
• Emotion/mood state
• Meal/nutrient consumption
• Dietary salt/water
• Stimulant use
Other important determinants
• Age
• Sex
• Disordered sleep
• Comorbidities (chronic renal disease, diabetes, heart failure, etc.)

ANS autonomic nervous system, *RAAS* renin–angiotensin–aldosterone system

The usually higher awake BP derives primarily from the dominance of sympathetic tone, verified by highest concentrations of plasma norepinephrine and epinephrine achieved after awakening (Lakatua et al. 1986). The high-amplitude circadian rhythms in the constituents of the RAAS—prorenin, plasma renin activity, angiotensin-converting enzyme (ACE), angiotensin I and II, and aldosterone—also play a prominent role (Angeli et al. 1992), independent of alteration of posture with daytime activity and nighttime sleep (Kool et al. 1994). On the other hand, the normal lower BP during nighttime sleep relative to daytime wakefulness represents the simultaneous impact of not only several predicable-in-time cyclic behavioral and environmental influences, but also circadian stage-dependent phenomena, most prominently, decline of sympathetic and rise of vagal tone, depression of the RAAS in the first half of sleep (followed by progressive activation during the second half of rest until peaking in the morning), and elevation of certain vasoactive peptides,

e.g., calcitonin and atrial natriuretic and calcitonin gene related (Angeli et al. 1992; Bartter et al. 1979; Gordon et al. 1966; Kanabrocki et al. 2001; Portaluppi et al. 1992, 2012; Sothern et al. 1995; Smolensky et al. 2007; Winters et al. 1988).

Various circadian rhythms in physiologic and biochemical functions and processes may also significantly affect the pharmacokinetics (PK) and pharmacodynamics (PD) of BP-lowering medications. Indeed, the PK of hypertension medications are significantly affected by the well-documented circadian rhythms in gastric pH, transport, and emptying; gastrointestinal motility; biliary function; glomerular filtration; hepatic enzyme activity; and organ blood flow, e.g., duodenum, liver, and kidney (Bélanger et al. 1997; Bruguerolle and Lemmer 1993; Gupta et al. 1995; Koopman et al. 1989; Labrecque and Beauchamp 2003; Okyar et al. 2012; Reinberg and Smolensky 1982). Significant and clinically meaningful ingestion-time (relative to the circadian time structure) differences have been documented in the PD of hypertension medications in both therapeutic effects on the daily BP pattern and adverse effects (Hermida et al. 2007d, 2011a, 2013c, 2014b, c; Smolensky et al. 2010, 2012). These result from circadian rhythm of drug PK but also in drug-free fraction, metabolic/clearance, and receptor number/conformation, second messengers, and signaling pathways of drug-targeted sites, e.g., heart, kidney, ANS, and RAAS (Hermida et al. 2007c; Smolensky and Haus 2001; Smolensky and Portaluppi 1999; Smolensky et al. 2012; Witte and Lemmer 2003).

This chapter presents the latest findings pertaining to the chronotherapy of hypertension—the judicious scheduling of conventional BP-lowering medications in accord with circadian rhythm determinants—as a simple and cost-effective means of lowering cardiovascular disease (CVD) and stroke risk, accomplished by normalizing altered characteristics of the 24 h BP pattern that are most significantly correlated with and deterministic of elevated risk of end-organ injury and fatal and nonfatal vascular events (Hermida et al. 2011b, 2012a, 2013b).

11.2 Differential Prognostic Value of Certain Features of the 24 h BP Pattern

The diagnosis of hypertension and all clinical decisions regarding its treatment typically are based on a limited number of daytime BP measurements obtained at the clinic, occasionally supplemented by wake-time patient self-assessments at home and work (Mancia et al. 2013). However, the correlations between BP level and risk of target organ damage and CVD and stroke events are higher for ambulatory BP monitoring (ABPM) (Clement et al. 2003; Eguchi et al. 2008; Hermida et al. 2011b, 2012a, b, 2013j; Perloff et al. 1983; Salles et al. 2008; Verdecchia et al. 1994). An important advantage of around-the-clock ABPM is thorough description and quantification of the daily BP variation.

Specific features of the daily BP pattern have been explored as biomarkers or mediators of target tissue injury and triggers of and risk factors for CVD events—

angina pectoris, myocardial infarction, cardiac arrest, sudden cardiac death, severe arrhythmias, pulmonary embolism—and cerebrovascular events—ischemic and hemorrhagic stroke (Hermida et al. 2011b, 2013b, 2014a; Portaluppi and Hermida 2007; Portaluppi et al. 2012). Numerous studies consistently substantiate an association between the atypical and abnormal physiologic feature of blunted sleep-time relative BP decline (non-dipper/riser BP pattern) and increased incidence of fatal and nonfatal CVD events, not only in hypertensive patients (Astrup et al. 2007; Boggia et al. 2007; Brotman et al. 2008; Dolan et al. 2005; Eguchi et al. 2008; Hermida et al. 2011b, 2013b; Ingelsson et al. 2006; Kario et al. 2001; Nakano et al. 1998; Ohkubo et al. 2002; Salles et al. 2008; Sturrock et al. 2000; Verdecchia et al. 1994) but also in normotensive individuals (Hermida et al. 2013f). Furthermore, various independent prospective studies demonstrate CVD events are better predicted by the asleep than awake or daily BP means (Ben-Dov et al. 2007; Boggia et al. 2007; Bouhanick et al. 2008; Dolan et al. 2005; Fagard et al. 2008; Fan et al. 2010; Hermida et al. 2011b, 2012a, b, 2013b; Kikuya et al. 2005; Minutolo et al. 2011).

Ohkubo et al. (2002) performed a population-based study that investigated the prognostic value of a single baseline 24 h ABPM. After an average follow-up of 9.2 years, they reported that each 5 % blunting of the sleep-time relative SBP decline of hypertensive patients corresponded to 31 % increase in CVD mortality risk. Of particular relevance is their finding that the hazard ratio (HR) of CVD mortality of dipper hypertensives (HR = 2.37) and non-dipper normotensives (HR = 2.16) did not differ (Ohkubo et al. 2002). Further analysis after 10.8 years of follow-up by the simultaneous inclusion of both the baseline nighttime and daytime SBP values in the same Cox survival model revealed only the nighttime SBP significantly predicted CVD mortality risk (Kikuya et al. 2005). Fagard et al. (2008) conducted a meta-analysis on BP data of four prospective European studies, representing 3468 hypertensive patients; daytime and nighttime SBP means predicted all-cause and CVD mortality, coronary heart disease, and stroke, independent of clinic BP. However, when these same ABPM-derived daytime and nighttime means were entered *simultaneously* into survival models, the nighttime SBP mean predicted all outcomes, whereas the daytime SBP mean contributed no further prognostic precision, rendering nighttime SBP as the only independent marker of CVD outcome.

Overall, these studies demonstrate that elevated sleep-time BP constitutes a significant CVD risk factor, independent of daytime clinic BP measurements or ambulatory awake and 24 h BP means. Nonetheless, the findings and conclusions of most previous ABPM studies may be imprecise because of inherent limitations of their investigative methods. All previous studies addressing the merit of ABPM for predicting CVD risk, except the Monitorización Ambulatoria para Predicción de Eventos Cardiovasculares (MAPEC Study, i.e., Ambulatory Blood Pressure Monitoring for Prediction of Cardiovascular Events) discussed below (Ayala et al. 2013b; Hermida 2007; Hermida et al. 2010d, 2011b, c, d, 2012b, 2013b, e, f, g) relied upon only a single, low-reproducible (Hermida et al. 2002b, 2007b, 2013d) 24 h ABPM evaluation per participant at study inclusion. Such a study

design is unsound because it presumes all features of the baseline-determined ambulatory BP pattern are maintained without alteration during the many years of follow-up, despite BP-lowering therapy, aging, and/or development of target organ damage and concomitant morbidity. Additional limitations of most previous ABPM studies are (1) frequent use of arbitrarily fixed clock hours to define morning awakening and evening bedtime, resulting in daytime and nighttime BP means that do not accurately represent the true awake and asleep BP ones, because they are calculated without assessing and taking into account the actual rest and activity spans of each participant; and (2) analysis of the prognostic value of dipping status and nighttime BP mean without proper adjustment for the daytime BP mean. Moreover, lack of systematic and multiple ABPM evaluations of patients over time in all previously reported long-term follow-up studies precluded the opportunity to explore the potential reduction in CVD risk associated with modification of prognostic parameters by hypertension therapy, i.e., either increase of sleep-time relative BP decline toward a more normal dipper patterning or, more specifically, reduction of asleep BP mean, a relevant issue that is still a matter of open debate. Incorporation of periodic (at least annual) ABPM evaluations during follow-up, as in the MAPEC Study, clearly establishes that (1) features of the daily BP pattern change over time; and (2) therapeutic reduction of the asleep BP mean and increase of the sleep-time relative BP decline toward normal dipping lessen CVD risk (Hermida et al. 2010d, 2011b, c, d, 2012a, b, 2013b).

11.3 Sleep-Time BP as a Therapeutic Target for CVD Risk Reduction

Potential reduction in CVD risk through modification of the prognostic ABPM parameters by a time-specified hypertension-treatment strategy has so far been investigated only in the MAPEC Study, a prospective, randomized, open-label, blinded endpoint trial designed to test the hypothesis that bedtime hypertension chronotherapy exerts better ambulatory BP control and CVD risk reduction than standard therapy, i.e., all prescribed hypertension medications ingested in the morning. Complete details of the rationale and design of the MAPEC Study are reported elsewhere (Hermida 2007; Hermida et al. 2010d, 2011b, c, d, 2012b, 2013b, e, f, g). Briefly, 3344 subjects with baseline ABPM ranging from normotension to sustained hypertension were prospectively followed for a median duration of 5.6 years. Hypertensive participants at baseline were randomized to two treatment strategies: (1) all prescribed hypertension medications ingested upon awakening or (2) the complete daily dose of $\geq$1 of them ingested at bedtime. The treatment protocol forbid division of any prescribed once-a-day medications as a split dose, i.e., half ingested upon morning arising and half at bedtime. At baseline and thereafter at yearly intervals (more frequently if hypertension treatment required adjustment based on ABPM criteria), ambulatory BP and physical activity

(wrist actigraphy to accurately derive the awake and asleep BP means on an individual basis) (Crespo C et al. 2012, 2013) were simultaneously monitored for 48 h. Registered events included all-cause mortality, myocardial infarction, angina pectoris, coronary revascularization, heart failure, lower-extremity acute arterial occlusion, retinal artery thrombotic occlusion, hemorrhagic and ischemic stroke, and transient ischemic attack.

In the MAPEC Study, clinic BP *does not* independently predict CVD events when the outcomes model is adjusted for the asleep BP mean, the strongest predictor of CVD events among all potential contributing BP parameters (HR = 1.50, 95 %CI [1.37–1.63], $P < 0.001$ for each 1-SD elevation in asleep SBP mean; HR = 1.09 [0.98–1.22], $P = 0.089$ for each 1-SD elevation in clinic SBP). The best Cox regression model [fully adjusted for the significant influential characteristics of sex, age, diabetes, anemia, and chronic kidney disease (CKD)] includes only the asleep SBP mean (HR = 1.23, 95 %CI [1.16–1.32], $P < 0.001$) and the sleep-time relative SBP decline (HR = 0.98 [0.97–0.99], $P = 0.019$). Most important, when the asleep SBP mean is adjusted for the awake SBP mean, only the former significantly predicts CVD outcomes (HR = 1.63 [1.44–1.85], $P < 0.001$, for each 1-SD elevation in asleep SBP mean; HR = 0.94 [0.81–1.08], $P = 0.348$, for each 1-SD elevation in awake SBP mean).

To further investigate the clinical relevance of the asleep BP mean on CVD risk, the studied population of the MAPEC Study was divided into four groups according to BP level at the final ABPM evaluation, i.e., normal or elevated, using the established ABPM thresholds of 135/85 mmHg for the awake SBP/DBP means and of 120/70 mmHg for the asleep SBP/DBP means (Mancia et al. 2013; Hermida et al. 2013j), independent of clinic BP. Results indicate (1) equivalent adjusted HR of participants with normal asleep BP mean whether the awake BP mean is normal or elevated [$P = 0.489$ for total CVD events (Fig. 11.1, top); $P = 0.980$ for major CVD events—composite of CVD death, myocardial infarction, plus ischemic and hemorrhagic stroke—(Fig. 11.1, bottom)]; (2) equivalent HR in hypertensive patients with elevated asleep BP mean, independent of awake BP mean ($P = 0.385$ for total CVD events; $P = 0.099$ for major CVD events); and (3) significantly higher adjusted HR of CVD events in hypertensive patients with elevated compared to those with normal asleep BP mean, whether the awake BP mean is below or above 135/85 mmHg (Hermida et al. 2011b, 2012b, 2013b). In summary, the asleep, but not the awake, BP mean constitutes a highly significant *independent* prognostic indicator of CVD morbidity and mortality (Hermida et al. 2011b, 2013b).

Data from the MAPEC Study, in which participants were repeatedly evaluated by periodic 48 h ABPM, also allows prospective evaluation of the impact of changes in clinic and ambulatory BP during follow-up on CVD risk. Table 11.2 summarizes the results of the time-dependent Cox regression analysis (adjusted for age, sex, diabetes, anemia, CKD, baseline BP, and hypertension treatment) for total CVD events in the MAPEC Study (Hermida et al. 2013b). Progressive treatment-induced lowering of the awake, asleep, and 48 h means of SBP and DBP, but not clinic BP, is associated with significantly increased CVD event-free survival.

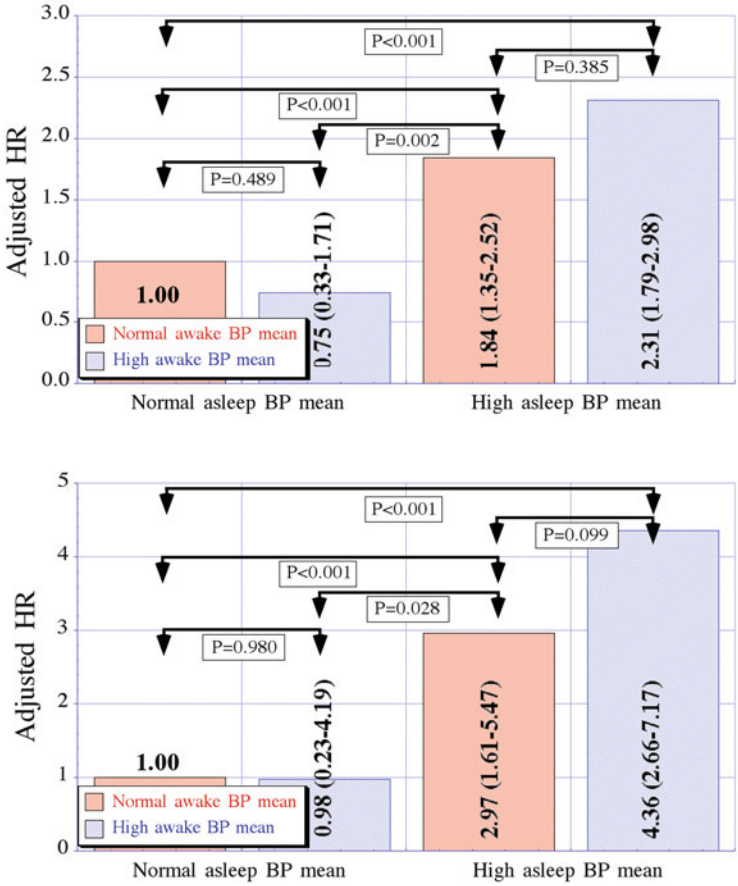

Fig. 11.1 Adjusted HR of total (*top*) and major CVD events (*bottom*) in the MAPEC Study. Major events included CVD death, myocardial infarction, and stroke. Participants were categorized into groups according to the level (normal or elevated) of the ABPM-derived awake and asleep SBP and DBP means. The awake SBP/DBP mean was considered normal if <135/85 mmHg and elevated if greater or equal. The asleep SBP/DBP mean was considered normal if <120/70 mmHg and elevated if greater or equal. Adjustments were applied for sex, age, diabetes, CKD, sleep duration, and hypertension treatment time—all medications upon awakening versus the entire daily dose of ≥1 medications at bedtime. Updated from Hermida et al. (2012b, 2013b)

Comparison of the results using standardized units (Table 11.2, right column) reveals that reduction from baseline in the asleep SBP/DBP mean is the most significant predictor of survival among all the tested ambulatory and clinic BP parameters. Most importantly, when the treatment-induced changes during follow-up in the asleep and awake BP means are entered jointly in the same time-dependent Cox regression model, progressive attenuation of the asleep SBP mean is significantly associated with increased event-free survival (adjusted HR = 0.65 [0.55–0.77], $P < 0.001$, for each 1-SD reduction in asleep SBP mean), while

Table 11.2 Adjusted HR of total CVD events in the MAPEC Study associated with reduction in clinic and ambulatory BP during median follow-up of 5.6 years

Parameter	Raw units	Standardized units
SBP		
Clinic	0.97 (0.93–1.01)	0.88 (0.77–1.03)
Awake mean	0.88 (0.83–0.93)*	0.72 (0.63–0.83)*
Asleep mean	0.83 (0.79–0.88)*	0.64 (0.56–0.73)*
48 h mean	0.85 (0.80–0.90)*	0.67 (0.58–0.77)*
Sleep-time relative decline	0.76 (0.69–0.85)*	0.69 (0.60–0.80)*
DBP		
Clinic	0.94 (0.88–1.02)	0.88 (0.76–1.03)
Awake mean	0.85 (0.77–0.94)**	0.76 (0.65–0.89)**
Asleep mean	0.73 (0.67–0.80)*	0.60 (0.52–0.69)*
48 h mean	0.78 (0.70–0.86)*	0.67 (0.57–0.78)*
Sleep-time relative decline	0.77 (0.70–0.84)*	0.65 (0.56–0.76)*

Hazard ratio (HR) (95 % CI) for each 5 mmHg decrease in BP and 5 % increase in sleep-time relative BP decline during follow-up (left column). Right column provides HR standardized by calculating them for 1-SD change in any given ABPM parameter during follow-up. Adjustments were applied for the influential characteristics of age, sex, diabetes, anemia, CKD, baseline BP, and number of prescribed hypertension medications. Change in BP was entered as a time-dependent covariate in the Cox regression models. Sleep-time relative BP decline, index of BP dipping, is defined as percent decline in BP during nighttime sleep relative to mean BP during daytime activity, and calculated as: ([awake BP mean − asleep BP mean]/awake BP mean) × 100. *$P < 0.001$; **$P < 0.01$. Updated from Hermida et al. (2013b)

progressive attenuation in the awake SBP mean is not (adjusted HR = 0.99 [0.86–1.14], $P = 0.849$, for each 1-SD decrease in awake SBP mean during follow-up). Thus, a diminished asleep, but not awake, BP mean is a highly significant independent prognostic marker of reduced CVD morbidity and mortality risk (Hermida et al. 2011b, 2013b).

11.4 Ingestion-Time Differences in Effects of Hypertension Monotherapies on Ambulatory BP

Many clinical trials now document reduction of asleep BP and the corresponding effects on the daily BP pattern, i.e., increasing the sleep-time relative BP decline toward a more dipper profile, by BP-lowering medications of six different classes are greatly improved when consistently ingested at bedtime than upon awakening (Hermida et al. 2007d, 2011a, 2013c, h, 2014b, c; Smolensky et al. 2010, 2012). Table 11.3 reports changes from baseline in the awake and asleep BP means plus sleep-time relative BP decline when hypertension medications of the different classes [ACE inhibitors (ACEIs), angiotensin-II receptor blockers (ARBs), calcium-channel blockers (CCBs), α-blockers, ß-blockers, and diuretics] are ingested either upon awakening or at bedtime by patients adhering to a normal

Table 11.3 Comparison of changes from baseline (in mmHg) in awake and asleep SBP/DBP means and sleep-time relative SBP/DBP decline by hypertension medications and their combinations when ingested upon awakening versus bedtime by hypertensive patients adhering to a normal routine of daytime activity and nighttime rest

Medication	Dose (mg)	Patients	Effect on awake SBP/DBP mean		Effect on asleep SBP/DBP mean		Effect on sleep-time relative SBP/DBP decline	
			Awakening R_x	Bedtime R_x	Awakening R_x	Bedtime R_x	Awakening R_x	Bedtime R_x
ACEIs								
Ramipril	5	115	−10.1/−6.9	−10.5/−9.0	−4.5/−4.1	−13.5/−11.5*	−3.3/−1.8	3.4/4.9*
Spirapril	6	165	−9.9/−8.0	−8.5/−5.7	−5.7/−4.6	−12.8/−8.6*	−2.5/−2.7	4.1/4.5*
ARBs								
Valsartan	160	90	−17.0/−11.1	−12.0/−9.8	−15.9/−10.8	−17.9/−13.3	0.2/1.3	5.4/6.3*
Valsartan	160	100[a]	−12.8/−6.6	−13.0/−8.5	−10.9/−5.5	−20.5/−11.1*	−1.0/−0.3	6.6/5.4*
Valsartan	160	200[b]	−13.1/−8.3	−12.6/−9.3	−12.9/−8.1	−21.1/−13.9*	0.4/0.9	7.2/7.1*
Olmesartan	20	133	−14.5/−12.1	−13.3/−9.6	−11.2/−8.7	−15.2/−11.5***	−1.3/−1.4	2.9/4.6*
Olmesartan	40	72	−17.1/−10.1	−16.3/−11.5	−12.6/−8.2	−17.9/−12.5***	−1.6/−0.2	3.0/3.8*
Telmisartan	80	215	−11.7/−8.8	−11.3/−8.2	−8.3/−6.4	−13.8/−9.7*	−1.6/−1.0	3.1/3.9*
CCBs								
Amlodipine	5	194	−10.2/−7.7	−11.8/−7.2	−9.6/−5.5	−11.2/−6.7	0.1/−1.6	0.2/0.7***
Nifedipine GITS	30	238	−9.4/−6.3	−12.8/−7.7***	−7.5/−5.1	−12.8/−7.8 *	−0.7/−0.2	1.0/1.5***
ß-blocker								
Nebivolol	5	173	−14.7/−12.4	−13.4/−10.9	−7.9/−7.4	−10.2/−8.1	−3.6/−3.0	−1.2/−1.4***
Diuretic								
Torasemide	5	113	−7.3/−3.7	−15.6/−9.9*	−4.3/−2.5	−12.5/−8.0*	−1.6/−0.7	−1.3/−0.2
α-blocker								
Doxazosin GITS	4	39[c]	−2.9/−3.7	−6.0/−5.4	0.7/−1.3	−8.2/−6.5**	−2.3/−2.4	1.9/1.9***
Doxazosin GITS	4	52[d]	−3.4/−2.9	−5.9/−4.4	0.1/−0.5	−4.9/−5.3***	−2.3/−2.4	1.7/1.5***
Combination R_x								

Valsartan/ amlodipine	160/5	203	−18.3/−14.5	−22.6/−12.7	−14.4/−10.1	−28.1/−14.7*	−1.3/−2.1	5.5/5.2*
Valsartan/ hydrochlorothiazide	160/12.5	204	−17.4/−11.5	−16.7/−11.4	−16.0/−12.0	−20.1/−13.6***	0.5/2.4	3.9/4.7*

All cited studies were conducted by some of the authors and followed a prospective, randomized, open label, blinded endpoint design. Participants (2306 patients in total) of all studies were grade 1 or 2 essential hypertension individuals, evaluated simultaneously by 48 h ambulatory BP monitoring and wrist actigraphy before and after timed treatment to accurately derive the awake and asleep SBP/DBP means and sleep-time relative BP decline (calculated as ([awake BP mean − asleep BP mean]/awake BP mean) × 100), i.e., the percent decline in mean BP during nighttime sleep relative to mean BP during daytime activity

Statistical significance of the comparison of effects on BP between treatment times: $^{*}P < 0.001$; $^{**}P < 0.01$; $^{***}P < 0.05$

[a]Study on elderly patients (≥60 years of age)

[b]Study on non-dipper patients, i.e., sleep-time relative SBP decline <10 %

[c]Patients treated with doxazosin monotherapy

[d]Patients treated with doxazosin in combination with other hypertension medications (polytherapy)

routine or daytime activity and nighttime sleep. The reported findings are derived from randomized, open-label, blinded endpoint trials, totaling 2306 investigated hypertensive patients, simultaneously assessed by 48 h ABPM at 20–30 min intervals and wrist actigraphy (activity level) at 1-min intervals to accurately derive the awake and asleep SBP/DBP means (Crespo C et al. 2012, 2013) and their dipper BP patterning on an individual basis before and after timed treatment. Table 11.3 shows that bedtime, compared to upon-awakening, ingestion of most tested BP-lowering medications resulted in statistically significant enhanced asleep BP mean reduction without loss of efficacy for reducing the awake BP mean, thus increasing the sleep-time relative BP decline.

11.4.1 Angiotensin-Converting Enzyme Inhibitors

A substantial number of studies entailing ACEIs [for an extensive review, see Hermida et al. (2013c)] demonstrates better BP-lowering effects upon the asleep than awake BP means, thereby converting the daily BP profile toward or into the normal dipping one, and/or improved safety when benazepril, captopril, enalapril, imidapril, lisinopril, perindopril, quinapril, ramipril, spirapril, trandolapril, or zofenopril are routinely ingested in the evening, preferably at bedtime, than upon awakening (Hermida et al. 2007d, 2011a, 2013c, h, 2014b, c; Smolensky et al. 2010, 2012). For example, a study of 33 untreated hypertensive patients documented that bedtime, compared to awakening, dosing of zofenopril (30 mg once daily for 1 month) better reduced the asleep BP mean, thereby increasing the proportion of patients with controlled ambulatory BP from 51.5 to 84.8 % ($P < 0.001$) (Balan et al. 2011). A larger clinical trial (Hermida and Ayala 2009) on 115 hypertensive patients randomized to either an upon-wakening or bedtime ramipril monotherapy regimen (5 mg once daily for 6 weeks) and assessed by 48 h ABPM before and after therapy found no ingestion-time-dependent difference in the awake SBP/DBP means, but very significant, larger reduction of the asleep SBP/DBP means by the bedtime compared to the morning-time schedule (−13.5/−11.5 vs. −4.5/−4.1 mmHg, $P < 0.001$ between groups; Table 11.3; Fig. 11.2, top). Another trial (Hermida et al. 2010a), utilizing an identical investigative protocol, involving 165 hypertensive patients randomized according to ingestion time of the long terminal plasma half-life (~40 h) spirapril (6 mg once daily for 12 weeks) also documented bedtime treatment best decreased the asleep SBP/DBP means relative to upon-awakening treatment (−12.8/−8.6 vs. −5.7/−4.6 mmHg; $P < 0.001$ between treatment-time groups; Table 11.3; Fig. 11.2, bottom) and most increased the sleep-time relative BP decline toward more dipper patterning.

The differential administration-time-dependent effects of ramipril and spirapril, two ACEIs with a markedly different plasma terminal half-life (Hermida et al. 2013c), on SBP are illustrated in Fig. 11.3. Ramipril reached peak effect sooner when ingested at bedtime than upon morning awakening, giving rise to significantly greater efficacy during the first 6 h following its ingestion (Fig. 11.3,

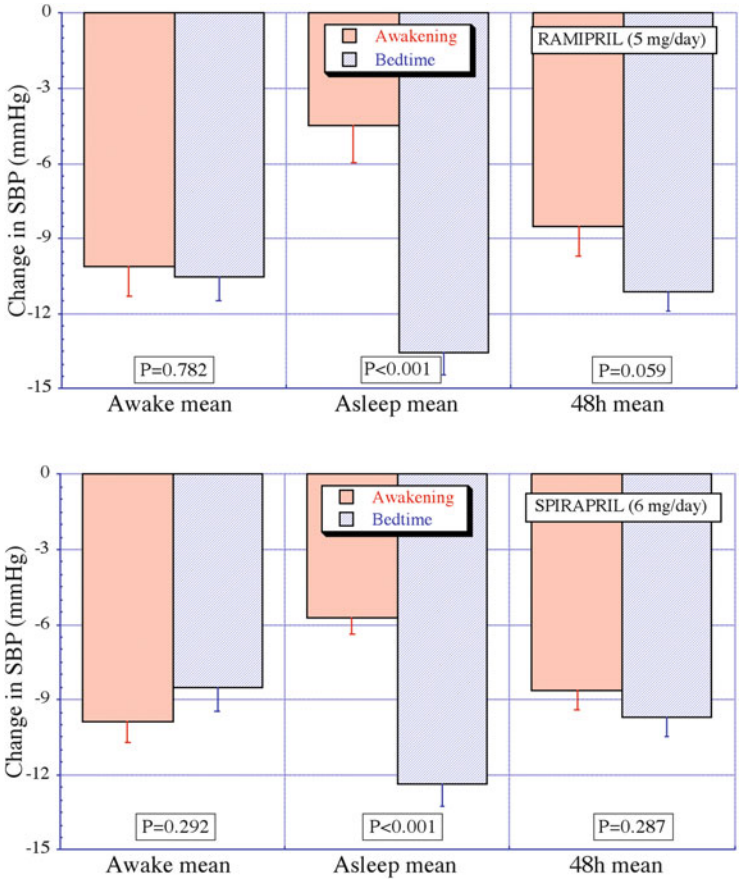

Fig. 11.2 Changes from baseline (mmHg) in awake (daytime activity span), asleep (nighttime rest span), and 48 h mean of SBP with ramipril (5 mg/day; *top*) and spirapril (6 mg/day; *bottom*) ingested upon awakening or at bedtime in patients with grade 1–2 essential hypertension studied by 48 h ABPM before and after several weeks of timed treatment. Probability values are shown for comparison of effects between the two treatment-time groups of patients by *t* test. Updated from Hermida and Ayala (2009) and Hermida et al. (2010a)

top). Moreover, the duration of the BP-lowering effect was shorter when ramipril was ingested upon awakening than at bedtime, resulting in BP reduction with bedtime ramipril administration being significantly greater during the last 12 h of the 24 h dosing interval (Hermida and Ayala 2009). The effects of the longer-half-life spirapril on SBP as a function of its ingestion time (Fig. 11.3, bottom) are surprisingly similar to those described for the shorter-half-life ramipril (Fig. 11.3, top). When ingested in the morning, spirapril starts losing its BP-lowering efficacy shortly after reaching peak effect, ~3 h after its ingestion. In contrast, when dosed at bedtime, maximum BP-lowering effect is maintained for 8 h after ingestion, thus showing greater efficacy, relative to morning administration, during this time

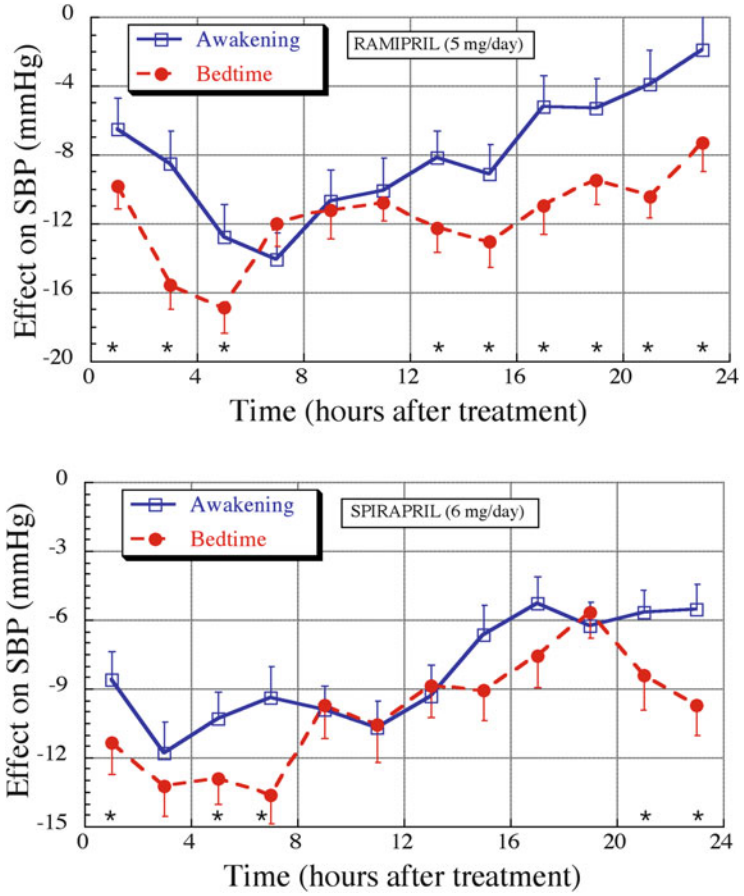

Fig. 11.3 Changes from baseline (mmHg) during the 24 h in SBP after treatment with ramipril (5 mg/day; *top*) and spirapril (6 mg/day; *bottom*) ingested either upon awakening or at bedtime. $*P < 0.05$ in BP reduction between the two treatment-time groups. Updated from Hermida and Ayala (2009) and Hermida et al. (2010a)

interval. Thus, the efficacy of spirapril, like ramipril, was significantly greater during the last half, particularly the last 4 h, of the dosing interval with bedtime, as compared to upon-awakening, administration (Hermida et al. 2010a).

11.4.2 Angiotensin-II Receptor Blockers

Clinical trials with ARBs also validate ingestion time-dependent effects of irbesartan, olmesartan, telmisartan, and valsartan (Hermida et al. 2011a, 2013c). Regardless of medication plasma half-life, the extent of reduction of the awake SBP/DBP means

did not differ with treatment time; however, the amount of reduction of the asleep SBP/DBP means was always significantly greater with bedtime treatment (Hermida et al. 2003, 2005b, d, 2007a, e, 2009; Pechère-Bertschi et al. 1988), thereby significantly lowering the prevalence from baseline of non-dipping. Hermida et al. (2003) assessed the efficacy of valsartan monotherapy (160 mg once daily for 12 weeks) when ingested either upon awakening or at bedtime by 90 hypertensive patients. When valsartan was ingested at bedtime, attenuation of the asleep SBP/DBP means was significantly greater than that of awake SBP/DBP means (respectively, −17.9/−13.3 vs. −12.0/−9.8 mmHg; $P = 0.009/0.015$; Table 11.3). Consequently, the bedtime schedule resulted in a highly significant average increase by 6 % in the sleep-time relative BP decline, which translated into a 73 % reduction from baseline in the number of non-dipper patients (Hermida et al. 2003). These results are corroborated by two subsequent independent prospective trials, the first conducted on elderly hypertensive patients (Hermida et al. 2005d), who as a group are characterized by greater blunting of the sleep-time relative BP decline than younger hypertensive patients (Hermida et al. 2013a; Jumabay et al. 2002; O'Sullivan et al. 2003). The second was conducted on non-dipper hypertensive patients (Hermida et al. 2005b, 2007a). In this later group, reduction of the asleep SBP/DBP mean was significantly greater when valsartan was ingested at bedtime than upon awakening (respectively, −21.1/−13.9 vs. −12.9/−8.1 mmHg; $P < 0.001$ between treatment-time groups; Fig. 11.4, top). The differential effects of 160 mg/day valsartan on SBP as a function of the time of drug ingestion for the composite of all the above three presented trials (Hermida et al. 2003, 2005b, 2007a) are shown in Fig. 11.5 (top). Significant lowering of SBP during the entire 24 h dosing interval was achieved independent of medication ingestion time. However, SBP reduction was significantly greater during the first 10 h following treatment when valsartan was ingested at bedtime.

Telmisartan, contrary to valsartan, has a long terminal plasma half-life (≥24 h) (Sharpe et al. 2001). Accordingly, it has been postulated that telmisartan would be effective in reducing BP homogenously throughout the entire 24 h independent of treatment time and without alteration of the circadian BP pattern (Neutel and Smith 2003). In order to test this hypothesis, Hermida et al. (2007e) studied 215 hypertensive patients randomized to either upon-awakening or bedtime telmisartan monotherapy (80 mg once-daily for 12 weeks). Significant and comparable lowering of the awake SBP/DBP means from baseline was achieved by both schedules [−11.7/−8.8 mmHg after treatment upon awakening vs. −11.3/−8.2 mmHg at bedtime; $P > 0.505$ between treatment-time groups (Table 11.2 and Fig. 11.4, bottom)]. The bedtime schedule, however, was significantly more effective in decreasing the asleep SBP/DBP means [−13.8/−9.7 vs. −8.3/−6.4 mmHg; $P < 0.001$ between treatment-time groups (Table 11.3 and Fig. 11.4, bottom)]. As a consequence, the sleep-time relative SBP/DBP decline was slightly changed toward non-dipper patterning when telmisartan was ingested upon awakening (−1.6/−1.0, $P = 0.010/0.157$), while it was significantly enhanced and better

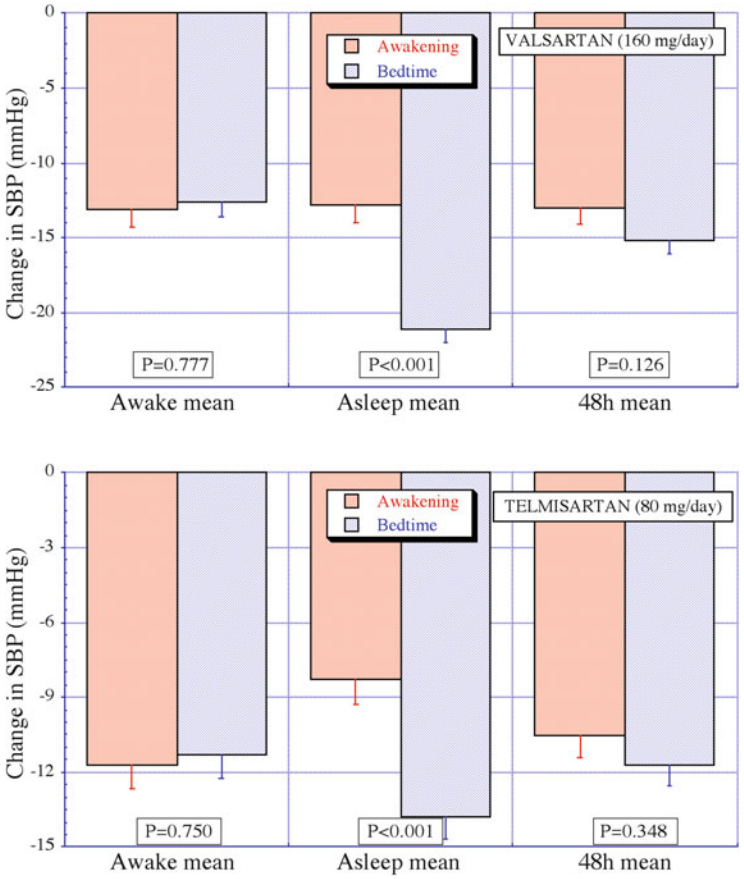

Fig. 11.4 Changes from baseline (mmHg) in awake (daytime activity span), asleep (nighttime rest span), and 48 h mean of SBP with valsartan (160 mg/day; *top*) and telmisartan (80 mg/day; *bottom*) ingested upon awakening or at bedtime in patients with grade 1–2 essential hypertension studied by 48 h ABPM before and after 12 weeks of timed treatment. Probability values are shown for comparison of effects between the two treatment-time groups of patients by *t* test. Updated from Hermida et al. (2007a, e, 2013c)

normalized when ingested at bedtime [3.1/3.9, $P < 0.001$ (Table 11.3)], thereby attenuating the prevalence from baseline of the non-dipper BP pattern by 76 % ($P < 0.001$) (Hermida et al. 2007e).

The differential effects of telmisartan on ambulatory BP relative to its administration time are shown in Fig. 11.5 (bottom). Despite its long half-life, when telmisartan was ingested in the morning upon awakening, but not when ingested at bedtime, it progressively lost its BP-lowering efficacy 16 h after ingestion. Consequently, BP reduction was significantly greater during the last 8 h of the dosing interval when the medication was ingested at bedtime. Additionally, treatment efficacy on BP was significantly greater during the first 2–8 h after bedtime

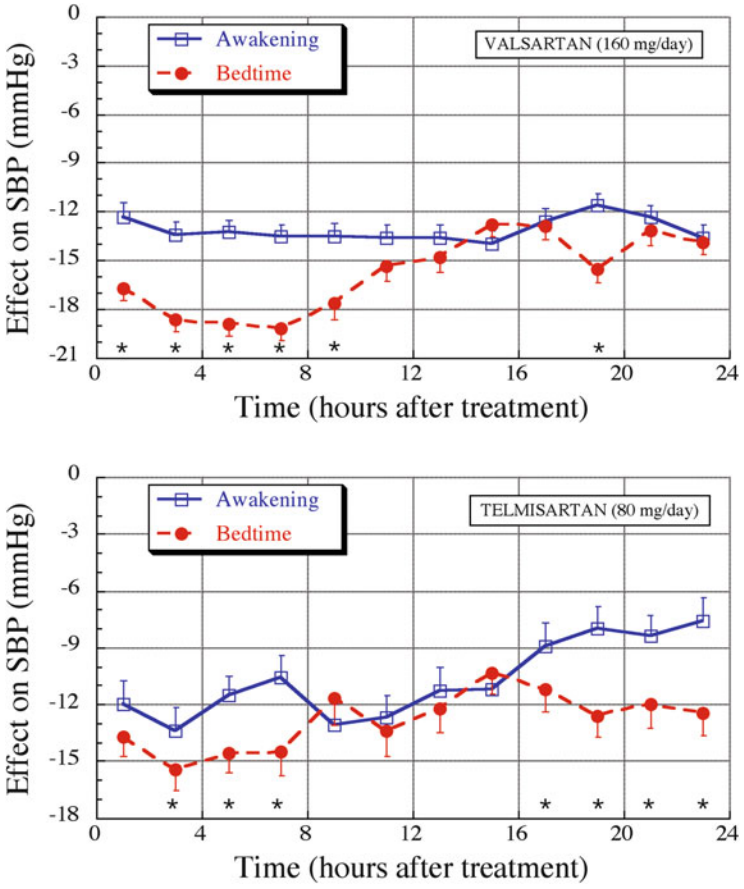

Fig. 11.5 Changes from baseline (mmHg) during the 24 h in SBP after treatment with valsartan (160 mg/day; *top*) and telmisartan (80 mg/day; *bottom*) ingested either upon awakening or at bedtime. $*P < 0.05$ in BP reduction between the two treatment-time groups. Updated from Hermida et al. (2007e, 2013c)

dosing (Fig. 11.5, bottom). The poorer BP-lowering efficacy during the last 8 h of the 24 h dosing interval following telmisartan ingestion upon awakening, corresponding to the nighttime sleep span of most investigated participants, and its greater efficacy during the first 8 h of the 24 h dosing interval following ingestion at bedtime, again representing the nighttime sleep span, are jointly responsible for the significant added efficacy of telmisartan in reducing the asleep BP mean with bedtime as compared to upon-awakening dosing (Fig. 11.5, bottom). These differential ingestion-time effects can be largely explained in terms of when, during the 24 h, highest and lowest drug concentrations are attained relative to the activation of the circadian rhythm in the RAAS, i.e., during nighttime sleep. The same explanation applies to the differential effects of ACEIs when ingested in the morning upon awakening versus at bedtime, as discussed in the previous section.

Importantly, bedtime, but not morning, ingestion of valsartan (Hermida et al. 2005c), olmesartan (Hoshino et al. 2010), and candesartan (Eguchi et al. 2012; Kario et al. 2010) significantly lessened urinary albumin excretion, which correlated strongly with decreased asleep BP mean, increased sleep-time relative BP decline (Hermida et al. 2005c), and improved baroreflex sensitivity (Eguchi et al. 2012). The RAAS circadian rhythm, with peak activity toward the end of the nighttime sleep span, is the hypothesized explanation for the better BP regulation conveyed by the bedtime regimens of ACEIs and ARBs (Hermida et al. 2011a, 2014c).

11.4.3 Other Hypertension Monotherapies

CCB treatment-time investigation of amlodipine, cilnidipine, diltiazem, isradipine, nifedipine, nisoldipine, and nitrendipine reveals that dihydropyridine derivatives typically reduce BP homogeneously throughout the 24 h, whether consistently ingested in the morning or evening (Hermida et al. 2013c). Of clinical relevance are the findings of a randomized study of 238 hypertensive patients that showed bedtime versus upon-awakening nifedipine GITS monotherapy (30 mg once daily for 8 weeks) resulted in significantly reduced incidence of its most common and troublesome adverse effect, i.e., peripheral edema (1 vs. 13 %; $P < 0.001$) (Hermida et al. 2008c).

Other hypertension monotherapies, including the α-blocker doxazosin (Hermida et al. 2004), ß-blockers carvedilol (Koga et al. 2005) and nebivolol (Hermida et al. 2006), and loop-diuretic torasemide (Hermida et al. 2008b), also evidence significantly enhanced asleep BP reduction and longer duration of BP-lowering effect with the bedtime, as compared to upon-morning awakening, treatment schedule (Table 11.3) (Hermida et al. 2013c).

11.5 Ingestion-Time Differences in Effects of Fixed Combination Hypertension Therapies on Ambulatory BP

Most hypertensive patients require treatment with more than one BP-lowering medication to achieve target BP goals (Dahlöf 2009; Mancia et al. 2013; Milani 2005). Despite substantial evidence of ingestion-time differences in the effects of various classes of hypertension monotherapies, thus far only a small number of studies have entailed trialing of combination medications at different times of the day, as summarized below. In summary, combinations of hypertensive medications also display consistent advantage of the bedtime schedule, mainly in terms of enhanced reduction of asleep BP mean (Hermida et al. 2013c).

Middeke et al. (1991) first reported once-daily 25 mg captopril/12.5 mg hydrochlorothiazide combination therapy (13 hypertensive men for 3 weeks) was slightly more effective in reducing nighttime BP mean when ingested at 20:00 h and significantly more effective ($P < 0.01$) in reducing daytime BP when taken at 08:00 h. Meng et al. (2010) randomized 40 hypertensive patients whose BP was uncontrolled with either amlodipine or fosinopril monotherapy, into two groups for 4-week combination therapy with both medications—Group-A: morning (07:00–08:00 h) amlodipine (5 mg) and bedtime fosinopril (10 mg) ingestion or Group-B: morning ingestion of both medications together. Nighttime SBP/DBP means of Group-A patients were reduced by a substantially greater amount than those of Group-B patients (−22.4/−17.4 vs. −7.6/−6.3 mmHg, $P < 0.001$). Further, the sleep-time relative BP decline was increased in Group-A and decreased in Group-B participants, thereby converting the 24 h profile of Group-A, but not Group-B, toward normal dipper patterning. Zeng et al. (2011) examined the differential therapeutic effects of 12-week morning (08:00 h) or evening (22:00 h) fixed-dose, single-pill amlodipine (5 mg) and hydrochlorothiazide (25 mg) combination therapy in 80 hypertensive patients. Evening versus morning treatment significantly better lowered the nighttime BP mean, and thus better decreased (25 % vs. 8 %; $P < 0.001$) the number of patients with non-dipper BP patterning.

In a much larger and detailed combination therapy study, Hermida et al. (2010b) randomly assigned 203 hypertensive patients to four different 12-week valsartan (160 mg)/amlodipine (5 mg) combination therapy regimens: both medications upon awakening, both at bedtime, or either medication ingested upon awakening and the other at bedtime. Ingestion of both medications together at bedtime resulted in greatest reduction of asleep SBP/DBP means, thereby significantly increasing sleep-time relative BP decline toward more normal dipper patterning ($P < 0.001$; Table 11.3). Hoshino et al. (2010) conducted an open-label, randomized crossover study of the effects of the morning versus bedtime administration of amlodipine–olmesartan combination on a small group of 31 hypertensive patients. The bedtime, compared to morning, scheduling of the combination more effectively decreased nighttime BP in non-dipper but not dipper patients, while also lowering the urinary albumin/creatinine ratio (42.5 ± 59.9 mg/g vs. 75.3 ± 26.4 mg/g, $P = 0.044$). The authors concluded bedtime ingestion of amlodipine/olmesartan combination seems more appropriate than morning dosing to obtain the therapeutic goals of reducing nighttime BP and improving renal function.

Finally, Hermida et al. (2011e) evaluated 204 hypertensive patients with ambulatory BP uncontrolled to published ABPM criteria (Hermida et al. 2013j; Mancia et al. 2013) after initial randomization to valsartan monotherapy (160 mg once daily for 12 weeks) either upon awakening or at bedtime. Hydrochlorothiazide (12.5 mg) was added and administered as a single-pill combination formulation with valsartan, with patients maintaining for an additional 12 weeks the original awakening or bedtime treatment-time schedule. Bedtime, compared to upon-awakening, combination therapy better reduced the asleep means of SBP (20.1 mmHg vs. 16.0 mmHg, $P = 0.015$; Table 11.3) and pulse pressure, i.e., SBP–DBP, a measure of the compliance of the arterial tree (6.5 mmHg vs. 4.0 mmHg,

$P = 0.007$), while it also significantly reduced non-dipper BP patterning, from 59 % of patients at baseline to 23 % at study conclusion ($P < 0.001$).

11.6 Chronotherapy in Difficult to Control and Complicated Hypertension

11.6.1 Resistant Hypertension

Resistant hypertension (RH) constitutes a clear illustration of the clinical relevance of a chronotherapeutic strategy that takes into account circadian changes in the physiology and biochemistry of BP control and regulation. Hypertension is considered resistant to treatment when lifestyle measures and ingestion in therapeutic doses of ≥3 BP-lowering medications, one preferably a diuretic unless contraindicated, fail to reduce SBP and DBP to recommended clinic BP threshold criteria (Calhoun et al. 2008; Fagard 2012; Mancia et al. 2013), a definition we feel lacks clinical validity (Hermida et al. 2013j), as later discussed.

RH patients are at considerably greater risk for stroke, renal insufficiency, and CVD events than those whose BP is well controlled (Ayala et al. 2013a; Calhoun et al. 2008; Cuspidi et al. 2001). The currently recommended therapeutic strategies for treating RH entail prescription of additional medications or exchange of one for another in an attempt to achieve better synergy of effects (Calhoun et al. 2008; Fagard 2012; Mancia et al. 2013). Based upon the review presented in the preceding sections, which clearly substantiates enhancement of BP-lowering efficacy by a bedtime treatment schedule of both hypertension monotherapies and fixed combinations (Table 11.3), it is logical to question whether RH patients are said to be "resistant" to therapy because it is prescribed and ingested at the wrong rather than right circadian time, i.e., morning rather than bedtime, when most effective (Cugini et al. 1996; Hermida et al. 2005a, 2008a, 2010c, 2013i, j; Ríos et al. 2013).

One cross-sectional study by Hermida et al. (2005a) of 700 RH patients assessed by 48 h ABPM found the proportion of patients with controlled ambulatory BP (awake and asleep SBP/DBP means below current diagnostic thresholds) was twofold greater when the entire daily dose of ≥1 hypertension medications was routinely ingested at bedtime than when all medications were taken upon awakening. Additionally, the prevalence of non-dipping was significantly lower in patients ingesting ≥1 medications at bedtime than in those ingesting all of them on awakening, respectively, 57 % versus 82 % (Hermida et al. 2005a). A larger more recent identically designed cross-sectional study of 1794 RH patients also evaluated by 48 h ABPM (Hermida et al. 2010c) documented control of ambulatory BP among those ingesting the entire daily dose of ≥1 medications at bedtime was significantly higher (31.9 % of patients) than in those ingesting all medications upon awakening (23.1 %; $P < 0.001$). Moreover, the bedtime, versus upon-awakening, treated patients evidenced significantly lower asleep SBP/DBP means

(by 9.7/4.4 mmHg, $P < 0.001$), resulting in significantly greater (by 5.8 %; $P < 0.001$) sleep-time relative BP decline as well as significantly lower prevalence of non-dipper BP patterning (40 % vs. 83 %, respectively; $P < 0.001$).

Hermida et al. (2013i), using a cross-sectional cohort from the ongoing multicenter Hygia Project (Ayala et al. 2013b; Crespo JJ et al. 2013; Mojón et al. 2013; Moyá et al. 2013; Ríos et al. 2013), also investigated the impact of hypertension treatment-time regimen on the daily BP patterning of RH patients evaluated by 48 h ABPM. The Hygia Project, comprising patients of primary care centers of Galicia (Northwest Spain), prospectively evaluates the prognostic value of ABPM and hypertension treatment time on CVD risk. ABPM assessment is done upon recruitment and at least annually thereafter for 48 h, rather than 24 h, to increase the reproducibility of findings (Hermida et al. 2002b, 2007b, 2013d). Among the 2899 evaluated RH patients, 1084 were ingesting all hypertension medications upon awakening (awakening regimen), 1436 the full daily dose of $\geq$1 of them at bedtime (bedtime regimen), and 379 split doses of $\geq$1 medications twice daily, upon awakening and at bedtime (BID regimen). Bedtime, compared to the upon-awakening and BID treatment, regimen resulted in significantly higher prevalence of properly controlled ambulatory BP, lower asleep SBP/DBP means, higher sleep-time relative BP decline, and lesser prevalence of non-dipping (54.4 % vs. 80.5 and 77.3 %, respectively; $P < 0.001$ between treatment groups) (Hermida et al. 2013i).

The findings and conclusions of these cross-sectional RH studies have been prospectively validated in a randomized trial that evaluated the effect of treatment time, without an increase in the number of prescribed medications, on ambulatory BP pattern and control of 250 true RH patients, defined by uncontrolled awake and/or asleep ambulatory BP means, who at baseline were taking all three of their prescribed BP-lowering medications upon awakening (Hermida et al. 2008a). Participants were randomly assigned to one of two groups according to the designated modification of the treatment strategy: (1) Group-A: exchange of 1 of the 3 medications for a new one, and retaining the same upon-awakening ingestion schedule; and (2) Group-B: also exchange of 1 of the 3 medications for a new one, but always ingesting it at bedtime. 48 h ABPM studies conducted before and after 12 weeks of the new therapeutic schemes revealed for Group-A no change in ambulatory BP from baseline and slightly increased prevalence of non-dipping, from 79 % at baseline to 86 % at study conclusion ($P = 0.131$); and for Group-B significant reduction in ambulatory 48 h SBP/DBP means ($-9.4/-6.0$ mmHg; $P < 0.001$), with greater decrease of asleep than awake BP, so the proportion of patients displaying dipper patterning increased from only 16 % at baseline to 57 % at study conclusion ($P < 0.001$) (Hermida et al. 2008a). Recently, a small study of 27 RH patients by another group (Almirall et al. 2012) demonstrated shifting all non-diuretic hypertension medications from morning to evening not only significantly reduced nighttime BP ($P = 0.005$), but enhanced the sleep-time relative BP decline toward more normal dipper patterning.

Most important, among the RH patients randomized according to the time of treatment in the MAPEC Study (Hermida et al. 2010d), those who routinely ingested the entire daily dose of $\geq$1 hypertension medications at bedtime showed

significantly lower HR of total CVD events (adjusted for the variables of age, sex, and diabetes) than ones who ingested all their medications upon awakening (0.38, 95 %CI [0.27–0.55]; $P < 0.001$) (Ayala et al. 2013a). The difference between the treatment-time groups in the adjusted HR of major events (a composite of CVD death, myocardial infarction, ischemic stroke, and hemorrhagic stroke) was also statistically significant (0.35 [0.18–0.68]; $P = 0.002$). Based upon the currently available scientific evidence, bedtime ingestion of the entire daily dose of ≥ 1 BP-lowering medications constitutes the most cost-effective therapeutic approach yet of improving both BP control and CVD event-free survival of patients who when managed by a morning treatment approach are incorrectly said to be inherently, i.e., physiologically, resistant to BP control (Ayala et al. 2013a; Hermida et al. 2010d).

In summary, the findings of the above-reviewed studies demonstrate that a bedtime hypertension medication regimen, in conjunction with proper patient evaluation by ABPM to corroborate the diagnosis of true RH, according to its prevailing definition, is the therapeutic scheme of choice (Hermida et al. 2013j; Niskikawa et al. 2013). The collective findings, however, further suggest that the current definition of RH is invalid and must be modified to take into account the determinist variable of treatment time; accordingly, a patient should be categorized as resistant to treatment only if his/her ABPM-determined awake and/or (preferably) asleep SBP or DBP means are greater than the reference diagnostic thresholds (Hermida et al. 2013j; Mancia et al. 2013) when at least one of the prescribed ≥ 3 hypertension medications of different classes, ideally including a diuretic unless contraindicated, is ingested in complete daily dose at bedtime (Hermida et al. 2013j).

11.6.2 Chronic Kidney Disease

The prevalence of hypertension is quite high in chronic kidney disease (CKD), increasing with diminishing estimated glomerular filtration rate (eGFR), and according to one report being as high as 86 % in end-stage renal disease (Agarwal et al. 2003). Moreover, the prevalence of elevated asleep BP (sleep-time hypertension) and non-dipper BP patterning is also high in CKD (Agarwal and Andersen 2005; Crespo JJ et al. 2013; Davidson et al. 2006; Mojón et al. 2013; Pogue et al. 2009; Portaluppi et al. 1990). Mojón et al. (2013) assessed by 48 h ABPM a large cohort of 10,271 hypertensive participants in the Hygia Project including 3227 patients with CKD [defined as eGFR <60 ml/min/1.73 m^2, albuminuria (albumin/creatinine ratio ≥ 30 mg/gCr), or both, at least twice within a 3-month period (Kidney Disease 2013)], finding the prevalence of non-dipper BP patterning significantly higher in those with (60.6 %) than without CKD (43.2 %; $P < 0.001$ between groups). The prevalence of riser BP patterning (sleep-time relative SBP decline <0), which is associated with the highest CVD risk, constituted the greatest difference between cohorts (17.6 % vs. 7.1 % in patients with and without CKD,

respectively; $P < 0.001$). The proportion of patients with the riser BP pattern significantly and progressively increased from 8.1 % for stage-1 CKD to a very high 34.9 % for stage-5 CKD. Most important, 90.7 % of uncontrolled hypertensive participants with CKD evidenced sleep-time hypertension (Mojón et al. 2013). These collective findings constitute the rationale for testing bedtime chronotherapeutic strategies for CKD, as reviewed below, to improve the management of high BP and to curtail disease progression.

Crespo JJ et al. (2013) recently investigated the impact of hypertension treatment-time regimen on ambulatory BP control and patterning in CKD. Among the 2659 hypertensive participants with CKD, 1446 took all their BP-lowering medications upon awakening and 1213 others ingested the entire daily dose of ≥ 1 of them at bedtime. Among the latter, 359 patients ingested all such medications at bedtime, while 854 ingested the complete daily dose of some of them upon awakening and the others at bedtime. Patients managed with the bedtime regimens, relative to those managed with the upon-awakening one, exhibited significantly lower asleep SBP/DBP means and higher sleep-time relative BP decline ($P < 0.001$), thereby significantly decreasing the prevalence of non-dipping from 68.3 % in those taking all hypertension medications upon awakening to 54.2 % and 47.9 % in ones taking, respectively, at least one or all of them at bedtime ($P < 0.001$ between groups). Moreover, the prevalence of riser BP patterning was much lower in participants ingesting ≥ 1 (15.7 %) or all (10.6 %) hypertension medications at bedtime rather than all of them upon awakening (21.5 %; $P < 0.001$ between groups). Finally, patients of the cohort that took all their medications at bedtime showed significantly higher prevalence of controlled ambulatory BP ($P < 0.001$) that was achieved by a significantly fewer number of BP-lowering medications ($P < 0.001$) compared to the patients of the other treatment cohorts who achieved inferior BP control (Crespo JJ et al. 2013).

Minutolo et al. (2007) evaluated a rather small sample of 32 uncontrolled non-dipper CKD patients and reported significant reduction of the nighttime BP mean, with consequent decreased urinary albumin excretion, after shifting 1 - BP-lowering medication from morning to evening. The findings of Rahman et al. (2013) for 151 black participants enrolled in the African American Study of Kidney Disease with controlled clinic and awake BP differed somewhat. This study compared the effects on nocturnal SBP—improperly defined according to an identical fixed clock-hour span for all participants—of either shifting to bedtime of an already prescribed once-a-day hypertension medication or adding at bedtime a new low-dose one. Both strategies decreased nocturnal SBP, but not significantly ($P = 0.08$), prompting the authors to conclude bedtime chronotherapy might be of limited advantage in reducing nighttime BP in hypertensive African-American patients and/or CKD. One recent Nigerian study, however, involving 165 black hypertensives randomized to 12 weeks of morning (10:00 h) or evening (22:00 h) hypertension treatment revealed significantly greater reductions in BP and left ventricular mass ($P < 0.001$) among those treated at night (Okeahialam et al. 2011). Finally, a recent study of 60 non-dipper Chinese patients with CKD by Wang et al. (2013) found bedtime relative to morning valsartan (80–320 mg

once daily for 1 year) treatment was significantly more effective in lowering nighttime BP, albuminuria, and left ventricular mass (P always <0.05). Collectively, the results of these studies of CKD patients of different ethnicities indicate the broad application of the bedtime hypertension chronotherapeutic strategy. Due to the very high prevalence of abnormal 24 h BP profiling in CKD, i.e., sleep-time hypertension and non-dipping/rising BP patterning, plus the documented better effects of bedtime hypertension strategy on asleep BP regulation (Hermida et al. 2007d, 2011a, 2013c, 2014b, c; Smolensky et al. 2010, 2012), it has been recently recommended as the preferred therapeutic approach to manage hypertension in patients with CKD (Hermida et al. 2013h, j, 2014d).

11.6.3 Diabetes

There is strong association between diabetes and elevated risk of end-organ damage, stroke, and CVD morbidity and mortality. Non-dipping and sleep-time hypertension are highly prevalent in patients with diabetes (Ayala et al. 2013a; Cuspidi et al. 2006; Moyá et al. 2013; Pistrosch et al. 2007). Ayala et al. (2013a) compared the features of the ambulatory BP pattern of 2954 hypertensive patients with type 2 diabetes and 9811 hypertensives without diabetes enrolled in the Hygia Project. The prevalence of non-dipping was significantly higher in those with diabetes than without diabetes (62.1 vs. 45.9 %; $P < 0.001$); however, the largest difference between groups was the prevalence of riser BP patterning (19.9 vs. 8.1 % in patients with and without diabetes, respectively; $P < 0.001$). Additionally, 89.2 % of uncontrolled hypertensive patients with diabetes in this cohort evidenced sleep-time hypertension. Despite these findings, the consequence of hypertension treatment time on BP regulation in diabetes has being scarcely investigated.

Tofé and García (2009) used a crossover design to evaluate the ambulatory BP response of 40 hypertensive individuals with type 2 diabetes to olmesartan (40 mg once daily for 8 weeks) when ingested either upon awakening or at bedtime. Bedtime treatment resulted in both significantly greater reduction of nighttime SBP (-16.2 vs. -11.8; $P = 0.007$) and increase in sleep-time relative SBP decline (7.4 vs. 2.2 %; $P < 0.001$).

Moyá et al. (2013) investigated the differential beneficial effects of hypertension treatment-time regimen on ambulatory BP patterning of 2429 hypertensive participants in the Hygia Project with type 2 diabetes. Among them, 1176 took all BP-lowering medications upon awakening and 1253 the entire daily dose of ≥ 1 medications at bedtime—336 patients taking all hypertension medications at bedtime and 917 the entire daily dose of some of them upon awakening and others at bedtime. Ingestion of ≥ 1 medications at bedtime compared to ingestion of all medications upon awakening significantly better reduced the asleep SBP/DBP means and better normalized the sleep-time relative BP decline. Thus, the prevalence of non-dipping was significantly higher when all hypertension medications were taken upon awakening (68.6 %) than when ≥ 1 (55.8 %) or all of them (49.7 %;

$P < 0.001$ between groups) were taken at bedtime. The latter treatment group, relative to all the other ones, also showed significantly higher prevalence of properly controlled ambulatory BP ($P < 0.001$), which was achieved by a significantly lesser number of hypertension medications ($P < 0.001$) (Moyá et al. 2013).

Suzuki and Aizawa (2011) provide an example of the misleading conclusion that commonly results from poorly conceptualized protocols when applied to trials of chronotherapeutic strategies. These investigators randomized 34 already treated hypertensive patients with type 2 diabetes into three groups according to valsartan (160 mg) treatment plan: entire daily dose either after breakfast or after dinner or half the dose (80 mg) twice daily. The investigators reported no significant difference in reduction of clinic or home self-measured BP between groups. The findings of this and several other studies of administration-time differences in therapeutic effect of hypertension medications that relied upon daytime clinic cuff and/or home BP measurements are of little, if any, practical utility for many reasons. First, "white-coat" effects may compromise the representativeness of clinic BP measurements, while inconsistent technique of self-assessment and poor patient compliance may compromise the accuracy of home BP data. Second, choice of dosing times is based on clock time, rather than biological time relative to the individual patient sleep–wake routine. Third, and most important, the investigative protocol of these studies did not collect data throughout the entire 24 h to reliably derive the clinically meaningful characteristics of the daily BP profile that are closely linked with CVD risk, particularly the asleep SBP mean and sleep-time relative SBP decline. Thus, the findings of studies that use inadequate protocols are likely misleading and add confusion and uncertainty to the medical literature plus unnecessary controversy on how to best manage hypertensive patients.

11.6.4 Non-dipper Hypertension

Several chronotherapy trials have specifically addressed the control of asleep BP and/or increase of the sleep-time relative BP decline (dipping) of non-dipper hypertensive patients, in addition to the above-cited ones specifically entailing high-risk patients diagnosed with RH (Almirall et al. 2012; Hermida et al. 2005a, 2008a, 2010c, 2013i, j; Ríos et al. 2013), CKD (Crespo JJ et al. 2013; Minutolo et al. 2007; Rahman et al. 2013; Wang et al. 2013), and diabetes (Moyá et al. 2013; Tofé and García 2009), all these conditions being associated with a high prevalence of non-dipping.

In what might well be the first chronotherapy trial specifically conducted on non-dippers, Hermida et al. (2005b, 2007a) applied 48 h ABPM to assess the efficacy in 200 non-dipper hypertensive patients of valsartan (160 mg once daily for 12 weeks) when ingested either upon awakening or at bedtime. As expected from the earlier presentation of findings from treatment-time studies entailing ARBs, reduction of the asleep SBP/DBP mean was significantly greater in non-dipper patients routinely taking valsartan at bedtime than upon awakening

(Table 11.3), therefore resulting in significant increase in sleep-time relative BP decline and 75 % of patients reverting to normal dipper BP patterning. Further, bedtime-treatment scheduling of valsartan led to significant increase in the proportion of patients with controlled ambulatory BP plus significant decrease in urinary albumin excretion, a biomarker of renal function (Hermida et al. 2005b, 2007a).

A prospective, double-blind, placebo-controlled study by Qiu et al. (2005) involving 121 treated non-dipper hypertensive patients randomized to evening (22:00 h) 12.5 mg captopril or placebo treatment found that the ACEI both significantly reduced nighttime BP and restored normal BP dipping in 70 % of patients. However, this study lacked a morning-time treatment comparison group to enable proper appreciation of the findings. Takeda et al. (2009) studied 71 Japanese hypertensive patients ingesting long-active BP-lowering medications once daily in the morning. Shifting therapy of the 35 non-dipper patients to bedtime resulted in slight increase of the daytime SBP/DBP means (+5/+3 mmHg; $P < 0.02$) and marked decrease in nighttime means (−13/−6 mmHg, $P < 0.001$), thus enhancing the sleep-time relative SBP decline from 2.6 to 15.5 % ($P < 0.001$). Finally, Farah et al. (2013) investigated the role of treatment time on BP patterning of 60 - non-dipper hypertensive patients randomly assigned to continue the ingestion of their prescribed BP-lowering medications upon awakening or to shift the ingestion of all of them to bedtime. Significant reduction in nighttime BP mean occurred among patients transferred to bedtime therapy, with 86 % of them showing controlled ambulatory BP.

11.7 Influence of Bedtime Hypertension Chronotherapy on the Risk of CVD and Stroke Events

The published clinical trials reviewed in the preceding sections substantiate the advantage of conventional hypertension medications when used as a bedtime chronotherapy strategy, compared to the traditional morning-dosing approach, to better regulate asleep SBP/DBP means and normalize the 24 h BP profile even of difficult to control RH, CKD, type 2 diabetic, and non-dipper patients. This section, based on review of the findings from long-term outcomes trials, addresses the question of whether or not a bedtime hypertension strategy is more protective against nonfatal and fatal stroke and CVD events of hypertensive persons.

11.7.1 Heart Outcomes Prevention Evaluation Trial

The heart outcomes prevention evaluation (HOPE) trial tested the hypothesis that adding the ACEI ramipril versus placebo to already existing BP-lowering, cholesterol-reducing, and other preventive strategies significantly reduces CVD and

stroke events in a cohort of 9297 high-risk CVD patients ≥55 years of age (Yusuf et al. 2000). In this study, patients were also randomized to either vitamin E or placebo as another add-on medication. Although the Methods section of the HOPE trial publication (Yusuf et al. 2000) does not specify the ingestion time of the add-on placebo or ACEI therapies, oral presentations by the principal investigators at hypertension meetings and one of the associated publications state that they were consistently ingested at bedtime (Svensson et al. 2001). The results of HOPE outcomes trial established that the add-on *bedtime* ramipril, relative to placebo, therapy significantly reduced the primary outcome variables of death from CVD causes and new onset myocardial infarction and stroke plus the secondary ones of death from any cause, revascularization procedures, cardiac events, complications of diabetes, and hospitalizations for heart failure (Yusuf et al. 2000). Despite the very minor advantage of bedtime ramipril over placebo therapy in reducing *daytime* clinic SBP/DBP measurements (average of 3/2 mmHg), an around-the-clock ABPM study of a subsample of 38 HOPE participants indicates that the bedtime ramipril therapy exerted profound lowering effect on nighttime SBP/DBP (17/8 mmHg, $P < 0.001$ compared to placebo) that additionally resulted in a significant 8 % increase of the sleep-time relative BP decline (Svensson et al. 2001). The results of this small substudy with ramipril ingested at bedtime are consistent with those of the other investigations entailing a bedtime ACEI strategy for the management of hypertension, as summarized in Table 11.3 and reviewed above. Moreover, the findings of the HOPE trial are consistent with the expectation that bedtime hypertension therapy should be advantageous to reduce CVD risk. However, the HOPE trial protocol did not include a comparator morning ramipril treatment arm; therefore, it does not enable a test of the hypothesis that bedtime hypertension chronotherapy with a conventional medication best reduces CVD risk.

11.7.2 Syst-Eur and Syst-China Trials

Administration-time differences in the adverse effects of some classes of hypertension medications are also of great clinical importance in achieving clinical goals, which depends on patient adherence to therapy. Two examples are the Syst-Eur (Staessen et al. 1997) and Syst-China (Liu et al. 1998) outcome trials. These trials investigated whether evening dihydropyridine CCB nitrendipine therapy, compared to placebo, reduces stroke and other CVD complications in elderly patients with isolated systolic hypertension as diagnosed by in-clinic cuff BP measurements. The assumed rationale for the chosen treatment regimen seems to be the expected reduced risk of drug-induced peripheral edema and related patient discontinuations with evening versus morning CCB dosing, an assumption verified by the study conducted on nifedipine GITS some years later by Hermida et al. (2008c). In the Syst-Eur trial, 4695 patients were randomized to either nitrendipine or placebo; after 2 years of follow-up, active treatment reduced the primary endpoint of stroke

by 42 % ($P = 0.003$), plus CVD mortality by 27 % ($P = 0.07$) and total CVD outcomes by 31 % ($P < 0.001$) (Staessen et al. 1997). The almost identical protocol of the Syst-China trial entailing 2394 patients found after 3 years of follow-up that active treatment reduced total stroke events by 38 % ($P = 0.01$), total mortality by 39 % ($P = 0.003$), CVD mortality by 39 % ($P = 0.003$), stroke mortality by 58 % ($P = 0.02$), and total CVD outcomes by 37 % ($P = 0.004$) (Liu et al. 1998). Similar to the HOPE trial, the Syst-Eur and Syst-China trials did not assess the comparative effects on CVD and stroke risk of active treatment ingested in the morning.

11.7.3 Controlled Onset Verapamil Investigation of Cardiovascular Endpoints Trial

The controlled onset verapamil investigation of cardiovascular endpoints (CONVINCE) trial was designed to explore whether or not initial treatment with 180 mg of the unique formulation controlled-onset extended-release (COER) verapamil ingested at bedtime is equivalent to morning treatment with either 50 mg of the ß-agonist atenolol or 12.5 mg of the diuretic hydrochlorothiazide in preventing primary outcomes such as myocardial infarction, stroke, or CVD death (Black et al. 2003). The trial ended prematurely because the sponsoring pharmaceutical company, for commercial reasons, closed the trial 2 years earlier than planned; thus, the median follow-up of participants was only 3 years. At the end of the abbreviated 3-year follow-up period, there were no differences in primary outcome events between the two tested treatment strategies. Surprisingly, more CVD-related events occurred between 06:00 and 12:00 h, not only in those participants randomized initially to atenolol or hydrochlorothiazide, but also in those initially randomized to COER verapamil. Thus, the CONVINCE trial failed to substantiate any of the protective benefits against CVD events that were theorized to result through the specific attenuation of the morning BP, including its rapid rise upon commencement of diurnal activity.

In keeping with the apparently unexpected findings from the CONVINCE trial, the relationship between morning BP rise and CVD risk remains highly controversial. The extent of BP surge upon awakening has been associated with increased CVD morbidity and mortality in some, but not all, studies (Gosse et al. 2004; Hermida et al. 2011b, 2013b; Israel et al. 2011; Kario et al. 2003; Metoki et al. 2006; Verdecchia et al. 2012). The level of BP immediately after waking and the rate of BP rise coincident with the commencement of daytime activity have been hypothesized to be triggers for the documented higher incidence of stroke, myocardial infarction, and other CVD events at this time of day (Casetta et al. 2002; Chasen and Muller 1998; Cohen et al. 1997; Deedwania 1997; Deedwania and Nelson 1990; Elliot 1998; Gallerani et al. 1997; Manfredini et al. 1997; Mehta et al. 2002; Muller et al. 1989). However, the findings of prospective studies that investigated the prognostic significance of the morning BP surge are inconsistent.

In the MAPEC Study, a larger morning BP surge was associated with a significantly lower CVD risk, in line with the lower risk associated with increased dipping of the circadian BP pattern (Hermida et al. 2011b, 2013b). The largest risk was, indeed, found in subjects with a rising pattern (asleep BP mean greater than awake BP mean) and, thus, characterized by a negative morning BP surge, i.e., a BP reduction after awakening from nighttime sleep. This is similar to the findings on a small cohort of participants in the Syst-Eur trial evaluated by ABPM (Staessen et al. 1999). Additionally, Verdecchia et al. (2012) recently reported lowest CVD risk in hypertensive patients of the first quartile of the early morning BP surge determined by a single before-treatment 24 h ABPM evaluation of 3012 persons who were then followed for 8.4 years. Contrasting findings have been reported by others: a morning BP surge within the top decile was associated with a higher risk of stroke in the Jichii Medical School ABPM study (Kario et al. 2003) and of total CVD events in the Bordeaux hypertensive cohort study (Gosse et al. 2004). The results of the Japanese study by Kario et al. (2003), however, seem to be inconsistent with their reported higher prevalence of events among patients with a rising BP pattern in the same database (Kario et al. 2001). In the Ohasama study, a large morning BP surge was not associated with risk of total stroke events, but rather with a higher risk of cerebral hemorrhage (Metoki et al. 2006). In summary, bedtime ingestion of the special drug-delivery formulation of COER verapamil significantly reduces morning BP, but it exerts only a limited effect on asleep BP mean, as documented in one randomized trial showing twofold greater reduction in the awake than asleep SBP/DBP means (White et al. 1998). This being the case, an undesired consequence by this unique bedtime treatment strategy seems to be an increased prevalence of the higher CVD risk non-dipper BP patterning.

11.7.4 MAPEC Trial

As indicated above, the tested hypertension medications (ramipril, nitrendipine, and COER-verapamil) in these previous reviewed trials entailing evening therapeutic strategies were not randomized according to treatment time (awakening vs. bedtime); thus, these investigations cannot be considered as proper chronotherapy outcome trials. The MAPEC Study constitutes the first prospective trial specifically designed and conducted to completion to test the hypothesis that a bedtime hypertension chronotherapy that focuses specifically on the normalization of asleep BP mean and sleep-time relative BP decline better reduces CVD and stroke risk than conventional morning-time therapy (Ayala et al. 2013b; Hermida 2007; Hermida et al. 2010d, 2011b, c, d, 2012b, 2013b, e, f, g). After a median follow-up of 5.6 years, hypertensive patients randomized to ingest the entire daily dose of $\geq$1 BP-lowering medications at bedtime, in comparison to those randomized to ingest all prescribed hypertension medications upon awakening, as expected displayed significantly lower asleep BP mean, higher sleep-time relative BP decline, reduced prevalence of non-dipping (34 vs. 62 %; $P < 0.001$), and higher

prevalence of controlled ambulatory BP (62 vs. 53 %, $P < 0.001$). Most important, the bedtime therapy regimen, compared to the upon-awakening one, resulted in significantly lower adjusted HR of total CVD events (HR = 0.39, 95 %CI [0.29–0.51]; $P < 0.001$) and major CVD events—composite of CVD death, myocardial infarction, and ischemic and hemorrhagic stroke—(HR = 0.33 [0.19–0.55]; $P < 0.001$) (Hermida et al. 2010d). CVD risk was higher in patients randomized to treatment upon awakening, no matter the classes of BP-lowering medications ingested. Greater benefits were observed for bedtime than awakening treatment with ARBs (HR = 0.29 [0.17–0.51]; $P < 0.001$) and CCBs (HR = 0.46 [0.31–0.69]; $P < 0.001$) (Hermida et al. 2013e). However, patients randomized to ingest an ARB at bedtime, in comparison to any other class of medication, with or without additional hypertension drugs, evidenced significantly lowest HR of CVD events ($P < 0.017$) (Hermida et al. 2013e).

Thus, the MAPEC Study not only substantiates that the asleep SBP mean is the most significant prognostic marker of CVD morbidity and mortality (Hermida et al. 2011b, 2012a, b, 2013b), but it also substantiates that reduction of the asleep SBP mean by a hypertension treatment strategy consisting of ingesting the entire daily dose of $\geq$1 conventional BP-lowering medications at bedtime significantly and cost-effectively decreases CVD risk, both in patients of the general hypertension population (Hermida et al. 2010d) and in those of greater vulnerability and enhanced CVD risk diagnosed with CKD (Hermida et al. 2011d), type 2 diabetes (Hermida et al. 2011c, 2012a), and RH (Ayala et al. 2013a).

11.8 Conclusions

The goal of all hypertension treatment strategies is reduction of SBP and DBP with the objective to prevent end-organ injury and to decrease the risk of CVD, stroke, renal disease, and other life-threatening outcomes. The beneficial effect of BP lowering in terms of preventing CVD events is consistent and, to a certain extent, independent of the class of medications prescribed to achieve it, although this conclusion has been mainly derived from outcome trials targeting only daytime clinic BP and not other features of the daily BP pattern more strongly associated with increased CVD risk, such as the asleep SBP mean and sleep-time relative SBP decline. Current hypertension therapy strategies, almost exclusively focused upon attenuating daytime clinic BP (Mancia et al. 2013), have unfortunately not eliminated the CVD hazards associated with elevated BP; indeed, they have only succeeded in decreasing them by a markedly suboptimal ~33 % (Gradman 2011). Review of the CVD-event incidence of reported outcome trials reveals that a relatively low level of major CVD events has been achieved only in studies that specifically enrolled low-risk hypertensive patients, i.e., ones that avoided inclusion of higher risk patients with diabetes, CKD, previous CVD events, advanced organ damage, or who are elderly (Zanchetti 2009). The collective findings of past outcome CVD and stroke morbidity and mortality studies incorporating such

high-risk patients, managed by the conventional morning treatment strategy in the attempt to achieve published thresholds for daytime clinic BP (James et al. 2013; Mancia et al. 2013), indicate that hypertension treatment fails to sufficiently lower CVD risk, leading some to the questionable conclusion (Hermida et al. 2013e) that these patients have an inherent "residual CVD risk" that cannot be reduced or eliminated by BP-lowering treatment (Mancia et al. 2009).

The treatment strategies of all but a few previous outcome trials, and also those incorporated into the clinical practice of medicine, disregard the facts that the (1) correlation between BP and CVD risk is far stronger for ambulatory than clinic BP measurements (Clement et al. 2003; Eguchi et al. 2008; Hermida et al. 2011b, 2012a, b, 2013j; Perloff et al. 1983; Salles et al. 2008; Verdecchia et al. 1994); (2) ABPM-determined asleep SBP mean is an independent and superior predictor of CVD events compared to either the awake or 24 h BP means (Ben-Dov et al. 2007; Boggia et al. 2007; Bouhanick et al. 2008; Dolan et al. 2005; Fagard et al. 2008; Fan et al. 2010; Hermida et al. 2011b, 2012a, b, 2013b; Kikuya et al. 2005; Minutolo et al. 2011); and (3) BP-lowering efficacy and other beneficial effects on the daily BP pattern of six different classes of hypertension medications and their combinations exhibit statistically and clinically significant awakening versus bedtime treatment differences (Hermida et al. 2007d, 2011a, 2013c, h, 2014b, c; Smolensky et al. 2010, 2012). The findings of the MAPEC Study, based upon periodic systematic 48 h ABPM evaluation of all participants during a median follow-up of 5.6 years, constitute the first proof-of-concept evidence that the progressive reduction of the asleep SBP mean and correction of the sleep-time relative SBP decline toward the normal dipper BP profile, most efficiently accomplished by a bedtime hypertension treatment strategy, best attenuate the risk of CVD and stroke (Ayala et al. 2013b; Hermida et al. 2010d, 2011b, c, d, 2012b, 2013b, e, f, g). These results indicate that 24 h BP control and pattern normalization plus risk reduction are best achieved when hypertension medications are optimally timed in step with key physiologic, neuroendocrine, and other biological determinants. In this regard, it is noteworthy that the American Diabetes Association acknowledges the clinical relevance of this concept of hypertension chronotherapy by recommending hypertensive patients with diabetes to ingest one or more BP-lowering medications at bedtime (American Diabetes Association 2012). Future prospective long-term outcomes trials that incorporate periodic, annually or more frequent, ABPM assessments and simultaneous diary recording of bed and wake times—to accurately and reliably ascertain asleep and wake-time BP level and dipping status—as done in the recently completed MAPEC Study (Ayala et al. 2013b; Hermida et al. 2010d, 2011b, c, d, 2012b, 2013b, e, f, g) and currently ongoing Hygia Project (Ayala et al. 2013b; Crespo JJ et al. 2013; Hermida et al. 2013i; Mojón et al. 2013; Moyá et al. 2013; Ríos et al. 2013) are needed to confirm the beneficial effects (reduced risk of CVD events and target tissue and organ injury) and safety of enhanced sleep-time BP reduction by bedtime hypertension chronotherapy with conventional

medications. In the interim, we have recommended bedtime hypertension treatment be considered for improving the management of hypertension not only in diabetes but also in other high-risk persons with predominant sleep-time hypertension or non-dipper BP patterning, such as the elderly and those with RH and secondary hypertension, CKD, and obstructive sleep apnea (Hermida et al. 2013j).

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Chapter 12
The Circadian Clock as a Drug Target

Sadichha Situala, Ariadna Amador, and Thomas P. Burris

Abstract The 24-h circadian rhythm manages virtually all aspects of physiology. Aberrations in circadian function are associated with myriad disorders ranging from behavioral disorders to metabolic diseases and cancer. The molecular clock regulating the circadian rhythm has been characterized and is composed of a transcriptional/translational feedback loop that maintains the rhythm and communicates with various physiological systems to maintain their circadian timing. Various molecular components of the clock are "druggable" and recent research has begun to illustrate the potential utility of targeting the clock for treatment of human disease. Here, we review the components of the clock and the drugs that have been designed to modulate their activities as well as their potential utility as therapeutic agents.

Keywords Clock • Circadian rhythm • Metabolic disease • Cancer • Sleep disorders • Behavioral disorders • Drug discovery

12.1 The Basic Molecular Clock

Endogenous molecular clocks at the cellular level control the timing of physiological and behavioral activities and are promising targets of drug design and development. The clock is regulated by means of a transcriptional/translational feedback loop. The positive arm of the loop is formed by transcription factors BMAL1 (brain and muscle ARNTL-like 1/Arntl) and CLOCK (Circadian locomotor output cycles kaput), which bind to E-box containing genes and activate their transcription. PERIOD (PER) and CRYPTOCHROME (CRY), the molecular components of the negative arm of the loop, are E-box containing genes, which are thus modulated by the BMAL1/CLOCK transcription factor heterodimer (Fig. 12.1). After transcription and translation, they begin to accumulate in the cytoplasm of the cell and

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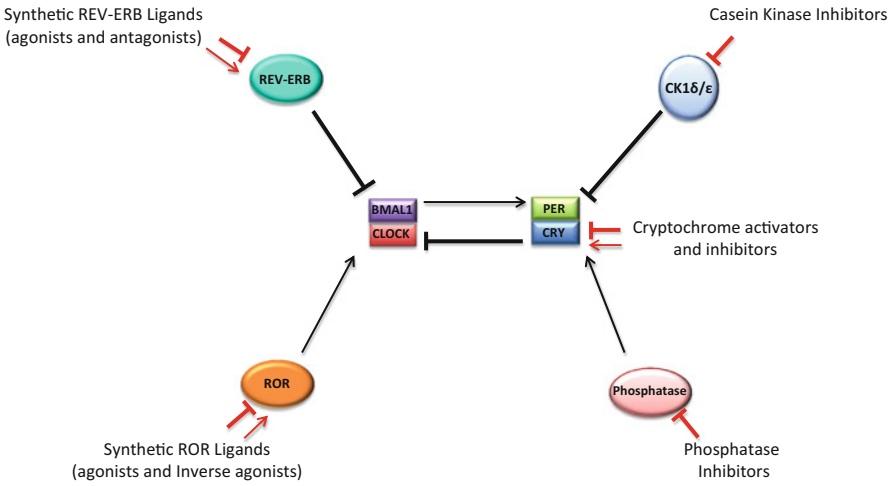

Fig. 12.1 Diagram of the molecular clock illustrating specific points where small molecule drugs have been designed to modulate the activity of the clock

translocate to the nucleus to block BMAL1 and CLOCK binding to DNA, resulting in the net reduction of PER and CRY levels (Reppert and Weaver 2002; Ko and Takahashi 2006; Schibler 2007). An interlocking loop is composed of "druggable" nuclear receptors REV-ERBα (encoded by *Nr1d1*) and REV-ERBβ (encoded by *Nr1d2*) (both E-box containing genes, and thus transcriptionally modulated by BMAL/CLOCK complex) and RORα,β,γ (encoded by *Nr1f1*, *Nr1f2*, *Nr1f3*). The REV-ERBs and retinoic acid receptor-related orphan receptors (RORs) recognize cognate DNA response elements known as the RORE (or RevRE) and either inhibit or activate, respectively, the transcription of their target genes. REV-ERB and ROR target genes include clock genes such as *Bmal1*, *Npas2*, and *Clock* (Fig. 12.1).

12.2 Why Target the Clock for Development of Therapeutics

The circadian rhythm regulates virtually every aspect of physiological function in humans and there is growing evidence that disruption of circadian rhythm is associated with adverse effects on human health. Altered circadian rhythms due to genetic, environmental, behavioral, or physiological factors contribute to the development of pathological conditions including metabolic syndrome, cancer, sleep disorders, psychiatric disorders, and inflammatory diseases.

Shift work has long been linked to disrupted circadian rhythm and sleep disorder. These workers have been reported to have higher incidence of cancer, psychological disorders, metabolic syndrome, and cardiovascular diseases (Schernhammer et al. 2003; Lund et al. 2001; Li et al. 2011; Knutsson et al. 1986, 1988; Kawachi

et al. 1995; Haus and Smolensky 2006; Karlsson et al. 2001; Davis et al. 2001). Jet lag and constant time zone change also affects the circadian clock and causes sleep disorder. Similarly, exposure to artificial light has been shown to disrupt clock rhythm (Fonken et al. 2013). These disorders resulting from circadian disruption due to environmental and behavioral factors are increasing as our lifestyle and work habits are constantly changing. Gene polymorphisms can also be responsible for sleep disorders. Genetic mutations in *PER2* and *CSNK1d* (encoding Casein Kinase 1δ) have been identified in humans with advanced sleep phase syndrome which is a condition where circadian clock is advanced by several hours (Toh et al. 2001; Vanselow et al. 2006; Xu et al. 2005).

The circadian clock regulates the expression of several genes involved in inflammatory processes. REV-ERBα is one of the important factors affecting the levels of inflammatory cytokines including Il-6 (Sato et al. 2014; Gibbs et al. 2012). Another component of the clock, RORα, is a strong mediator of inflammatory responses (Delerive et al. 2001). Genetic knockout (KO) studies in mice provide evidence for direct link between the autoimmune disease rheumatoid arthritis and core clock components. CRY1/2 double KO mice have disrupted circadian rhythm and these mice have more severe collagen-induced arthritis compared to wild-type mice (Hashiramoto et al. 2010).

Many studies have provided insights into the role of circadian clock in metabolic processes. Disruption in circadian clock components is associated with phenotypes of metabolic syndrome. Clock disruption is found to have adverse effects on energy homeostasis, lipid metabolism, and inflammation. Mice with genetic deficiency in clock components such as *Bmal1*, *Clock*, *Cry*, or *Rev-erb* display dyslipidemia, hyperglycemia, hyperinsulinemia, and obesity (Bass 2011, 2012; Turek et al. 2005; Green et al. 2008; Rudic et al. 2004; Bass and Takahashi 2010).

Circadian clock components are important regulators of cell cycle; thus, they would be considered potential players in diseases associated with abnormal cellular proliferation. Several polymorphisms in core clock genes have been associated with increased incidence of cancer. There is strong evidence supporting the role of PER proteins in different types of cancer. Mice deficient in PER2 develop lymphoma at a faster rate and rate of growth is decreased and apoptosis induction increased in cancer cells overexpressing PER1 or PER2 (Hua et al. 2006; Fu et al. 2002). In contrast to PER, loss of CRY proteins is shown to reduce the risk of cancer in mice (Ozturk et al. 2009).

Other conditions such as psychological disorders and cardiovascular defects have been increasingly linked to clock function. A better understanding of how clock components are coupled to the normal physiological processes may provide novel targets for the treatment of diseases associated with disrupted circadian clock. Several components of the circadian clock have been explored as possible targets for therapeutic intervention. Many drug-like compounds targeting clock proteins have been developed and are being studied, but there is an increasing need to gain a more detailed mechanistic understanding of clock-associated disorders and develop better therapeutics.

12.3 Drugging the Clock

As indicated above, the molecular clock is composed of multiple classes of proteins whose expression oscillates in a circadian manner. Some of these protein components belong to families that are proven targets for drug development such as the nuclear receptors and kinases. Others fall into protein families where there is little experience developing small molecule drugs that may be able to modulate their activities. Below, we describe each of the major components of the molecular clock along with reviewing the success (or lack thereof) in developing small drug-like molecules that can modulate their activity. We also review the effect of these clock-targeted drugs on the circadian rhythm as well as in disease models where data are available.

12.3.1 NPAS2/CLOCK:BMAL1

The transcription portion of the circadian core loop is majorly controlled by two bHLH (basic helix-loop-helix) and PAS (period-ARNT-single-minded) domain-containing transcription factors, CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle ARNT-like 1) (Dunlap 1999; Panda et al. 2002; Reppert and Weaver 2002; Zhang and Kay 2010). They belong to the bHLH family of transcription factors and are capable of forming heterodimers through HLH domain interactions. A PAS domain follows the bHLH structural motif and is formed by two adjacent repeats of about 130 amino acids. Although little is known regarding PAS domain function, it is understood that it confers specificity of partner choice to proteins in the bHLH/PAS family (Pongratz et al. 1998).

These bHLH transcription factors recognize E-box sites, the six-base pair conserved sequence CANNTG, mostly in a palindromic form as CACGTG (Murre et al. 1989a, b, 1994; Ephrussi et al. 1985; Ledent and Vervoort 2001; Swanson et al. 1995; Massari and Murre 2000). BMAL1 and CLOCK have been shown to bind to canonical and noncanonical E-boxes (E′-boxes), as is the case for PER2, CACGTT (Yoo et al. 2005). These transcription factors heterodimerize, bind to the consensus E-box DNA sequence, and result in the activation of transcription of their clock target genes (Gekakis et al. 1998; Hogenesch et al. 1998).

Crystallographic data have demonstrated that the CLOCK His84 and BMAL1 Leu125 are crucial for their bHLH mutual recognition. In addition, BMAL1 Ser78 appears to be the necessary amino acid for regulation of transcription inhibition (Wang et al. 2013). Interestingly, the PAS (Per-ARNT-Sim) domain is highly conserved, but can adopt different conformations upon interactions with different ligands (Moglich et al. 2009).

The first component of the molecular clock to be identified was CLOCK via a forward genetic approach (Vitaterna et al. 1994). The dominant negative CLOCK mutant ($CLOCK^{\Delta 19}$) lacks the protein transactivation domain (King et al. 1997).

Mice homozygous for CLOCK$^{\Delta 19}$ show longer circadian periods, with *tau* being about 27.3 h. Moreover, these mice turn behaviorally arrhythmic when housed in constant darkness (Lee et al. 2001). Nonetheless, in mice homozygous for a null mutant CLOCK, the rhythm within the suprachiasmatic nucleus and animal behavior remain unchanged (DeBruyne et al. 2006). The mice show a slightly shorter period when deprived of external cues. As CLOCK was identified to be a bHLH-PAS protein, it was expected to find a binding partner to perform as a regulator of circadian rhythms (Antoch et al. 1997; Kewley et al. 2004).

BMAL1 is a basic helix-loop-helix (bHLH)/PAS protein that was identified in a yeast two-hybrid screening as a CLOCK partner (Gekakis et al. 1998). It is expressed in different tissues, namely suprachiasmatic nucleus (SCN), eye, brain, liver, heart, kidney, skeletal muscle, and lung. Binding of BMAL1 to CLOCK results in their translocation to the nucleus where they bind to E-box genes and induce the transcription of clock targets, such as *Period* (*Per1*, *Per2*, and *Per3*) and *Cryptochrome* (*Cry 1* and *Cry 2*) genes, and nuclear receptors, *REV-ERB* and *RORα*.

BMAL1 contains a nuclear localization signal (NLS) and nuclear export signals (NES) in its N-terminal and PAS domains, respectively. BMAL1 then translocates into the nucleus from the cytoplasm allowing CLOCK nuclear accumulation (Kwon et al. 2006). In vitro experiments in HEK293 cells and fibroblasts deficient in BMAL1 showed that CLOCK intracellular localization, phosphorylation, and degradation depended on BMAL1 availability (Kondratov et al. 2003). Studies on *Bmal1* mutant mice have highlighted the role of this protein in the proper function of the molecular clock. *Bmal1*$^{-/-}$ mice were generated by deletion of its bHLH domain. These mice were found to be arrhythmic, which highlights the non-redundancy of this molecule in the molecular clock and its relevance in maintaining the 24-h cycle (Bunger et al. 2000).

NPAS2 (neuronal PAS domain protein), the surrogate of CLOCK in the SCN, compensates the lack of CLOCK in the brain as a BMAL1 transcriptional partner (DeBruyne et al. 2007). It is a member of the bHLH-PAS family of transcription factors. It has been found to uniquely express in the brain, colon, small intestine, and uterus (Zhou et al. 1997). Mice expressing a nonfunctional form of NPAS2 have a shortened period, and the CLOCK-deficient mice crossed with two alleles of nonfunctional NPAS2 show arrhythmic behavior when placed in constant darkness (DeBruyne et al. 2007).

There are no identified drugs directly targeting the NPAS2/CLOCK:BMAL1 complex, but CLOCK:BMAL1 heterodimers can be modulated in different manners. It is well established that CRY:PER are negative regulators of CLOCK:BMAL1 complex by directly interacting with them, forming a repressing complex containing all four molecules (Sato et al. 2006; Kiyohara et al. 2006; Lee et al. 2001; Chen et al. 2009). Interactions between CRY and CLOCK:BMAL1 complex occur via the PAS-B domain of CLOCK and the C-terminal region of BMAL1 (Zhao et al. 2007; Sato et al. 2006). The specific residues that determine interaction are Gly^{332}, His^{360}, Gln^{361}, Trp^{362}, and Glu^{367} in the CLOCK PAS-B domain (Huang et al. 2012).

12.3.2 Period

In mammals three Period (PER) proteins have been identified (Zylka et al. 1998). PER and CRY are a part of negative feedback loop within the molecular clock. These proteins form a repressor complex and inhibit the activity of CLOCK/BMAL1 in the circadian machinery. PER mRNA and protein levels display a circadian pattern of expression and their activity is regulated post-translationally by phosphorylation and dephosphorylation. The casein kinases (CK) CK1δ and CK1ε have been shown to phosphorylate PER proteins that leads to their cytoplasmic retention and protein degradation.

PER is a PAS domain-containing protein and contains several protein–protein interaction domains shown to be important for interaction with other PER proteins, CRY proteins, as well as the BMAL1/CLOCK heterodimer (Albrecht et al. 2007). It also bears binding sites for GSK3β kinase and ubiquitin ligase and contains CK1 phosphorylation sites. It has nuclear localization sequences in addition to a cytoplasm-localization domain (Albrecht et al. 2007). PER interacts with CRY proteins via the C-terminus (Albrecht et al. 2007; Rosato et al. 2001; Ozber et al. 2010). Interestingly, LXXLL motifs and CoRNR boxes, which are motifs for interaction with nuclear receptors and coregulators, are also present in the protein (Albrecht et al. 2007). PER2 proteins have been shown to directly interact with nuclear receptors including REV-ERBα, PPARα, NURR1, HNF4α alpha, and TRα (Schmutz et al. 2010; Ripperger et al. 2010).

The PER proteins have important functions in metabolism, stress response, brain, and sleep. In the brain, PER2 appears to have important role in feeding and addiction. In mice with a specific mutation in *Per2*, food anticipatory behavior was observed to be lost when animals were kept under a timed feeding schedule (Feillet et al. 2006). There is increasing evidence that *Per1* regulates alcohol-drinking behavior during stressful conditions. A study designed to evaluate the effect of stress on alcohol intake in *Per1* mutant mice lacking the PAS-binding domain demonstrated that Per1-deficient mice displayed enhanced alcohol consumption under stress (Dong et al. 2011). Interestingly, an association was found with frequency of drinking in adolescents with *PER1* promoter SNP rs3027172 (Dong et al. 2011). PER1 is known to be involved in stress response and was shown to be transcriptionally activated in corticotropin-releasing factor-expressing cells. *Per2* has also been implicated in sleep disorders. A form of familial advanced sleep phase syndrome where PER2 proteins harbor S662G mutations has been associated with 4-h advance of daily sleep–wake rhythm (Toh et al. 2001). This mutation is also linked to cell cycle progression and tumorigenesis (Fu et al. 2002; Hua et al. 2006; Gu et al. 2012).

Genetic loss-of-function studies have unraveled the role of PER proteins in circadian rhythm. *Per1* single KO mice display shorter circadian cycle (Bae et al. 2001) while loss of *Per3* does not appear to affect circadian rhythm significantly (Zheng et al. 2001). *Per2* appears to have a more dominant function in the

clock. *Per2*-deficient mice have a shorter period length in normal light–dark settings and become arrhythmic in complete darkness (Zheng et al. 2001). *Per1/Per2* double KO mice also display arrhythmic behavior (Bae et al. 2001).

There are no drugs reported to directly target PER proteins. Since these proteins are regulated by casein kinases, a number of kinase inhibitors, however, have been shown to modulate circadian rhythm by affecting the stability of PER proteins. For example, Longdaysin, an inhibitor of CK1δ activity, lengthened the period by inhibition of CK1δ-mediated phosphorylation and degradation of PER1 (Hirota et al. 2010). Mutations in the CK1δ binding site in PER2 are associated with advanced sleep rhythms. These data provide important information regarding the critical role of specific phosphorylation sites and potential of pharmacological modulation of phosphorylation and kinase activity to alter sleep cycle. This and other kinase inhibitors that mediate their effects through PER will be discussed below.

12.3.3 Cryptochrome

Two CRY genes are found in mammals, *Cry1* and *Cry2*, and they are critical components of the mammalian circadian clock forming a part of the core negative feedback loop. These proteins form repressor complex with PER proteins, translocate to the nucleus, and inhibit the activity of CLOCK/BMAL1 complex, consequently suppressing their own synthesis. A mammalian ubiquitin ligase, FBXL3, regulates the stability of CRY. Phosphorylation of CRY leads to the binding of CRY to FBXL3 and ubiquitination, which targets it for proteasomal degradation. Loss of FBXL3-dependent degradation has been shown to stabilize CRY proteins and lengthen circadian period (Siepka et al. 2007).

CRY proteins have important functions both inside and outside of clock. In addition to being a critical core loop component and maintaining circadian periodicity, CRY is shown to participate in cell cycle regulation by interacting with some cell cycle genes. CRY also participates in mediating cellular response to DNA damage by regulating checkpoint proteins such as TIMELESS (TIM) (Unsal-Kacmaz et al. 2005). Given these roles in cell cycle, it is not surprising that CRY is implicated in cancer. Indeed, loss of *Cry* reduced the risk of developing tumors in rodent models. The effect of *Cry1/2* mutation in p53-mutated mice was studied and it was showed that triple KO mice had lower incidence of cancer and longer life span (Ozturk et al. 2009). Other studies have shown that knockdown of *Cry* genes improves sensitivity to cancer chemotherapy by regulating tumor suppressor genes suggesting a novel pharmacological possibility for CRY inhibitors (Lee and Sancar 2011). CRY also plays a role in the regulation of metabolism and immune function (see Chaps. 5 and 9 for more on these topics). CRY binds to and represses glucocorticoid receptors that regulate the transcription of gluconeogenic genes.

Studies have shown the role of cryptochrome in the regulation of expression of proinflammatory cytokines. It was demonstrated that TNFα, IL-6, and iNOS levels were elevated in the hypothalamus of *Cry1* and *2* double KO mice and in *Cry1/2*$^{-/-}$ fibroblasts (Narasimamurthy et al. 2012). It was found that this constitutive cytokine activation in *Cry1/*$^{-/-}$ cells was due to the activation of NF-kappaB and PKA signaling pathway (Narasimamurthy et al. 2012). Studies in *Drosophila* revealed that *Cry* mRNA and protein levels are significantly reduced in older flies compared to younger ones. CRY-overexpressing flies maintained significantly higher climbing ability and lower oxidative damage compared to CRY-null flies (Rakshit and Giebultowicz 2013). These results suggest the potential interesting role of CRY in delaying behavioral and functional aging.

Previous studies have showed that single knockdown of *Cry1* leads to shortened period, while *Cry2* knockdown shows lengthened period (van der Horst et al. 1999; Thresher et al. 1998; Vitaterna et al. 1999). Double KO mice are found to be arrhythmic in constant darkness and display an aberrant metabolic phenotype (van der Horst et al. 1999; Vitaterna et al. 1999; Lamia et al. 2011). This is consistent with human genome-wide association studies that have identified association of *CRY* gene locus with fasting blood glucose levels and presentation of type 2 diabetes (Dupuis et al. 2010).

Given the physiological roles of cryptochromes and their implications in numerous disorders, studies have been initiated to target these proteins with the goal of developing novel therapeutics to treat clock-related and metabolic diseases. A chemical biology effort using high-throughput cell-based chemical screening identified KL001 as a small molecule that interacts specifically with CRY1/2 (Hirota et al. 2012). KL001 is a derivative of carbazole family of aromatic compounds and was shown to prevent ubiquitin-dependent degradation of CRY1/2 resulting in lengthening of the circadian period. The CRY1/2 stabilization mediated by KL001 inhibited glucagon-induced gluconeogenesis in primary hepatocytes in a dose-dependent manner suggesting potential utility in treating metabolic disorders such as type 2 diabetes (Hirota et al. 2012).

More recently, another group identified a small molecule modulator of both CRY1 and CRY2 using a two-step cell-based screening platform. Compound 15, a derivative of 2-ethoxypropanoic acid, was identified as a potent and efficacious small molecule candidate for CRY inhibition (Chun et al. 2014). While KL001 stabilizes CRY and represses core loop activity, compound 15 inhibits the repressive function of CRY1 and CRY2 and therefore activates E-box-mediated transcriptional activity. Compound 15 is shown to modulate clock-controlled gene transcription by inhibiting both CRY1/2 resulting in attenuation of rhythm. This compound does not affect the period. Mutation studies in C-terminal tail and putative CC domain showed that compound 15 selectively binds with these regions during its interaction with CRY proteins (Chun et al. 2014). This compound can be used to further explore the functions of CRY proteins and its role in metabolic disorders.

12.3.4 Retinoic Acid Receptor-Related Orphan Receptors

The three RORs (RORα,β,γ) belong to the nuclear receptor (NRs) family of ligand regulated transcription factors (Aranda and Pascual 2001; Beckerandre et al. 1993; Carlberg et al. 1994; Hirose et al. 1994). NRs are a family of transcription factors characterized by a highly conserved structure. This structure is organized into five regions: the "AB," "C," "D," "E," and "F"(1). "AB" is recognized as the N-terminus, which contains activation function 1 (AF-1), a region that is relatively non-conserved between members of the NR family and is associated with ligand-independent transcriptional activity in many receptors (Burris et al. 2013). The "C" region is a highly conserved region that contains two Zinc fingers and binds to the DNA; thus, it is also called DNA-binding domain (DBD). The "D" region constitutes a flexible linker or hinge that connects the DBD with the ligand-binding domain (LBD) and this region also plays a role in modulation of DNA binding. The "E" region is located at the C-terminal half portion and contains the LBD and helix 12 (H12). The structure of the LBD is conserved, although its sequence varies across NR family members. It is composed of 11–12 alpha helices organized into a three-layered sandwich-like structure with a single beta sheet that guard the ligand pocket. The LBD also contains the activation function 2 (AF-2) whose transcriptional activity is ligand dependent. H12 is crucial for transcription since it defines the binding of ligand, dimerization, and recruitment of transcriptional co-activators and corepressors. The "F" region is an extreme C-terminal region, is quite variable, is contained in only a limited number of NR family members, and its function is poorly understood.

All the RORs recognize the same DNA binding site known as an ROR response element or RORE. The RORE consists of a 6-bp motif AGGGTCA, flanked by an AT-rich 5′ sequence. A distinct class of ROREs may also appear in tandem repeats interspaced by two nucleotides (Forman et al. 1994; Harding and Lazar 1993, 1995). RORs act as activator of transcriptions from these sites. RORs were long thought to be "true" orphan nuclear receptor as they displayed constitutive transcriptional activation activity in the absence of any known ligand. However, it was initially suggested that cholesterol and cholesterol sulfate may be endogenous ROR ligands (Kallen et al. 2002) and newer studies have implicated various oxysterols and sterol metabolites as potential high-affinity endogenous ligands (Santori et al. 2015; Hu et al. 2015).

The patterns of expression of the three ROR are distinct. For example, RORα has been detected in lung, muscle, brain, heart, peripheral blood leukocytes, spleen, liver, and ovary (Hamilton et al. 1996a; Becker-Andre et al. 1993), whereas RORβ expresses exclusively in the brain, retina, and pineal gland (Becker-Andre et al. 1993; Schaeren-Wiemers et al. 1997). RORγ, on the other hand, exhibits a peripheral pattern of expression; it has been found to express in the thymus, muscle, testis, pancreas, prostate, heart, and liver.

The structure of the RORβ ligand-binding domain was first solved as RORβ bound to stearic acid using crystallographic methods (Stehlin et al. 2001). The

RORα LBD was crystallized bound to cholesterol and cholesterol sulfate (Kallen et al. 2004). Finally, RORγ was crystallized bound to oxysterol agonists 20α OHC, 22R-OHC, and 25-OHC and to the inverse agonist digoxin (Fujita-Sato et al. 2011). These structures provided insights into the conformational changes of RORs upon binding agonists and antagonists. Agonist-bound ROR facilitates co-activator binding via helix 12 (Jin et al. 2010). Nonetheless, the AF2 surface that contains H12 shifts away from its active conformation when ROR was bound to the inverse agonist. RORs interact with transcriptional co-activators, such as SRC1, SRC2, PGC1α, and p200/CBP, as well as transcriptional corepressors, such as NCOR1, NCOR2, RIP140, and NIX1 (Xie et al. 2005; Atkins et al. 1999; Liu et al. 2007; Yin and Lazar 2005; Poliandri et al. 2011; Greiner et al. 2000).

The function of the three RORs has been examined extensively in genetic models. As all isoforms of ROR bind to the same DNA-binding motif, it is predicted that RORs co-expressed in the same tissues may compensate for one another. Knockout and naturally RORα-deficient mice (staggerer mice) display cerebellar ataxia due to an apparent critical role RORα plays in Purkinje cell development (Hamilton et al. 1996b; Hadj-Sahraoui et al. 2001; Steinmayr et al. 1998). The staggerer mouse bears an insertion in the RORα gene, causing a phase shift and early transcriptional stop rendering RORα protein inactive. These mice display increased feeding, with lowered body fat and decreased susceptibility to developing steatotic liver. RORα involvement in glucose metabolism has been also described, mainly through targeting of *glucose-6-phosphatase* (*G6P*) (Chopra et al. 2008). Moreover, these mice are characterized by thin long bones, and the development of acute atherosclerosis when placed on a high-fat diet. Studies in single and double mutant RORα and RORγ mice revealed their role in regulating hepatic genes, such as 3β-hydroxysteroid dehydrogenases, cytochrome p450 enzymes, and sulfotransferases (Kang et al. 2007). The double mutant mice show reduced cholesterol, triglycerides, and glucose levels (Kang et al. 2007). RORγ$^{-/-}$ mice display increased rate of cellular apoptosis and lack of lymph nodes. Overexpression of RORγ is associated with inhibition of T-cell receptor-mediated apoptosis in T-cell hybridomas, of Fas ligand, and of interleukin 2. RORγ plays a role in glucose metabolism and regulates the expression of genes involved in fat mass, lipid homeostasis, and muscle activity (Raichur et al. 2010). RORα$^{-/-}$ mice as well as RORβ$^{-/-}$ mice exhibit changes in circadian behavior.

Several putative endogenous and synthetic ROR ligands have been described. The synthetic LXR agonist T0901317, which has also been shown to target FXR and PXR, has been shown to be a dual repressor of RORα/RORγ (Kumar et al. 2010; Mitro et al. 2007). From the T0901317 scaffold lead, other synthetic ROR ligands have been synthesized that have been shown ROR selectivity across the nuclear receptor panel. Among them, SR3335 is a RORα-selective inverse agonist (Kumar et al. 2011), SR1001 is a dual RORα/RORγ inverse agonist (Solt et al. 2011), SR1078 is a dual RORα/RORγ agonist (Wang et al. 2010b), and SR2211 is a RORγ-selective inverse agonist (Kumar et al. 2012).

Endogenous ligands identified for the RORs include stearic acid and all-trans retinoic acid modulating RORβ (Stehlin-Gaon et al. 2003; Stehlin et al. 2001).

Cholesterol and cholesterol sulfate were found to modulate RORα (Kallen et al. 2002, 2004). Finally, a plethora of putative endogenous ligands may modulate RORγ by direct binding including a range of sterols and oxysterols (Soroosh et al. 2014; Wang et al. 2010a, c; Jin et al. 2010; Santori et al. 2015; Hu et al. 2015).

12.3.5 REV-ERBs

REV-ERBs (REV-ERBα and REV-ERBβ) belong to the family of nuclear receptors as well. Unlike other nuclear receptors, REV-ERB lacks H12, which mediates binding with co-activators. However, this structural variation allows enhanced interaction transcriptional corepressors such as NCoR1. Like the RORs, REV-ERBs were originally thought to lack ligands, but this was due to their constitutive repressor activity (rather than activation activity in the RORs). However, we and others described heme as an endogenous ligand for the REV-ERBs in 2007 (Raghuram et al. 2007; Yin et al. 2007). Activation of REV-ERBs by agonists induces recruitment of corepressors leading to transcriptional repression of target genes. REV-ERBs bind to the very similar response elements as the RORs and there is considerable overlap with the genes they regulate. REV-ERBα and REV-ERBβ are widely expressed in a circadian manner and display considerable overlap in their patterns of expression.

REV-ERBs have a key role in the regulation of circadian rhythm as well as regulating other processes such as metabolic pathways, muscle biogenesis, glucose homeostasis, adipogenesis, and atherogenesis. Genome-wide studies have indicated that REV-ERB is required for circadian rhythm and lipid metabolism (Fontaine and Staels 2007; Duez and Staels 2008; Burris 2008). There is evidence supporting a wide range of important roles of REV-ERBα in lipid metabolism and it is also a well-characterized regulator of adipogenesis. It has been shown that the expression of SREBP1c and its target gene FAS is reduced upon silencing of REV-ERBα (Le Martelot et al. 2009). REV-ERBα KO mice display abnormally elevated triglyceride and VLDL levels (Raspe et al. 2002). Additionally, a connection between REV-ERBs and atherosclerosis was established with the finding that atherogenesis was increased in REV-ERBα KO mice (Ma et al. 2013) while a REV-ERB gain-of-function experiment displayed reduced atherogenesis (Sitaula et al. 2015). Apart from lipid homeostasis, REV-ERBs have also been shown to regulate hepatic glucose production and repression of gluconeogenic genes. Silencing of REV-ERBα in mouse islet cells was demonstrated to impair glucose-induced insulin secretion, decreased the expression of key lipogenic genes, and inhibited β-cell proliferation (Vieira et al. 2012). There is also a role for REV-ERB in muscle biogenesis. REV-ERBα is highly expressed in oxidative skeletal muscle and its deficiency in muscle resulted in reduced mitochondrial content and oxidative function leading to impaired mitochondrial biogenesis and increased clearance of mitochondria consequently resulting in lower exercise capacity and increased

skeletal muscle autophagy (Woldt et al. 2013). Other studies have shown REV-ERB association with cancer and its role in cell cycle (Kourtidis et al. 2010).

REV-ERBα deficiency in mice has been associated with developmental delays in cerebellum, delayed migration of granule cells, and increased apoptosis of neurons (Chomez et al. 2000). REV-ERBα/β double KO mice were shown to have profound alterations in circadian expression of core circadian clock and the genes involved in lipid homeostasis were also severely affected (Cho et al. 2012). In another study, the mice with deficiencies in expression of both REV-ERBs displayed lipid accumulation in liver (Bugge et al. 2012). This study showed that depletion of both REV-ERB α/β resulted in elevated hepatic triglyceride levels triggering hepatic steatosis (Bugge et al. 2012). These studies clearly emphasize the crucial roles of REV-ERBs in circadian rhythm and metabolism. Targeted activation of these receptors could hold utility in improving metabolic profile, altering physiological rhythms and energy homeostasis.

As indicated above, REV-ERBs were originally believed to lack ligands, but two studies demonstrated that heme binds with a K_d of 2–3 μM and increases recruitment of corepressor NCoR leading to ligand-dependent transcriptional repression (Raghuram et al. 2007; Yin et al. 2007). Heme levels are tightly regulated in cells and one of the mechanisms of this regulation is through negative feedback by REV-ERBs (Wu et al. 2009). The fact that heme has many biological functions and heme overloading can be toxic to cells prevented its use in exploring REV-ERB functions. However, the identification of heme as an endogenous ligand demonstrated that REV-ERBs are "druggable" and paved the way for the development of synthetic ligands as tools to investigate the biology of these receptors.

Soon after the recognition of heme as a REV-ERB ligand, efforts to develop the first synthetic REV-ERB agonists were initiated. A FRET-based assay monitoring the interaction of the REV-ERB LBD and interaction domain of NCoR successfully identified GSK4112 to target REV-ERBs. This small molecule was shown to promote NCoR recruitment, inhibit the expression of BMAL1, *Cry1*, and *Pgc1*, reduce gluconeogenic gene expression, and reduce glucose output in primary hepatocytes (Grant et al. 2010). These data showed that GSK4112 acts as a REV-ERB agonist and regulates expression of REV-ERB target genes in a manner similar to heme. This compound was valuable in the initial studies of REV-ERB in vitro, but the poor pharmacokinetic properties such as high clearance and rapid metabolism in the body made it a poor in vivo chemical tool. However, the information obtained in vitro was sufficient to prove that small molecule compounds such as this could be further optimized and used to characterize the biology of REV-ERBs.

These efforts were followed by the work from our group to design several REV-ERB agonists and antagonists. Two REV-ERB agonists SR9009 and SR9011 and the antagonist SR8278 were synthesized using GSK4112 as an initial hit compound (Solt et al. 2012; Kojetin et al. 2011). The REV-ERB agonists were demonstrated to inhibit the expression of BMAL1 and reduce the amplitude of the circadian oscillations in SCN explants (Solt et al. 2012). In vivo administration of the compounds showed reduced locomotor activity during the active period of mice

as well as altered amplitude and phase of core loop components (Solt et al. 2012). Moreover, these compounds greatly improved the metabolic profile of mice. Increased energy expenditure, reduced fat mass, reduced blood lipids and glucose, and reduced triglyceride synthesis were observed in mice treated with these agonists, suggesting that pharmacologic modulation of REV-ERBs using synthetic ligands is possible and could be used to regulate clock activity and treat metabolic disorders. A recent study has shown that treatment with a REV-ERB agonist improved mitochondrial function, increased endurance, and changed the skeletal muscle composition to an oxidative phenotype in mice (Woldt et al. 2013). Other studies have demonstrated reduced inflammatory reactions with REV-ERB agonist treatment (Gibbs et al. 2012; Sato et al. 2014). The REV-ERB agonist SR8728 has been demonstrated to stimulate the expression of gluconeogenic genes, but due to its poor pharmacokinetic properties, it has not been investigated in vivo. Another REV-ERB antagonist has been identified, cobalt protoporphyrin (identical to heme with the exception of the iron ion replaced with a cobalt ion), and its structure bound to the LBD of REV-ERB determined (Matta-Camacho et al. 2014). Newer studies with SR9009 and SR9011 as well as another agonist from a distinct scaffold with improved pharmacodynamics properties, SR10067, have shown that pharmacological activation of REV-ERB induces anxiolytic activity while also increasing wakefulness (Banerjee et al. 2014).

Further efforts to explore REV-ERB-dependent circadian and other functions with these drugs are ongoing; however, these compounds need to be further optimized. Improving the pharmacokinetic properties would mitigate the problem of a requirement of high dose to obtain biological effects in vivo. It should also be noted that SR9009 and SR9011 target both REV-ERBα and β, and to delineate the individual functions of the two REV-ERBs, subtype-specific ligands need to be developed. Several additional ligands based on GSK4112 have been optimized for potency, selectivity, and bioavailability in a recent study (Trump et al. 2013). While these medicinal chemistry efforts are adding to the tools available to investigate REV-ERB biology, such efforts may be more rapidly advanced by accompanied structural studies of the receptor.

12.3.6 Kinases

The kinase family of enzymes includes over 500 members and these proteins are involved in most cellular processes. The Casein Kinase (CK) belongs to a family of serine/threonine kinases and these kinases in particular are important modulators of posttranslational modifications in circadian clock components. Studies have shown that modifications by kinases are crucial in maintaining periodicity of the circadian clock. Specifically, kinases phosphorylate PER1 and PER2 and trigger proteasome-mediated degradation. This degradation releases the inhibition of BMAL1/CLOCK and allows the transcription of PER and CRY. CK1δ and CK1ε are of particular importance in regulating the stability and localization of PER proteins.

The CK1 family has seven mammalian isoforms and although CK1δ and CK1ε appear to have redundant function in the circadian pacemaker important differences in these kinases have been identified. Mutational studies have demonstrated that CK1δ has more dominant effects on the circadian period relative to CK1ε (Etchegaray et al. 2010; Meng et al. 2010; Walton et al. 2009). CK1δ deficiency has been shown to reduce PER protein turnover and have significant period-lengthening effect while CK1ε deficiency did not appear to have a major effect on period length or protein expression rhythms (Walton et al. 2009; Etchegaray et al. 2009; Meng et al. 2008).

Protein kinases have been targeted for the treatment of a number of diseases. Many inhibitors of CK1δ and CK1ε have been developed and tested for targeting the circadian clock. Small molecule inhibitors such as PF670462, IC261, D4476, PF4800567, and longdaysin have been shown to alter the circadian period and have allowed the study of the role of kinases and core clock proteins in physiological processes (Walton et al. 2009; Hirota et al. 2010; Long et al. 2012; Rena et al. 2004; Mashhoon et al. 2000). These inhibitors have period-lengthening effects that are suggested to be achieved through CK1δ/ε-mediated phosphorylation of PER proteins. PF4800567 is a CK1ε-specific inhibitor that shows minimal effect in period length regulation consistent with studies in CK1ε-deficient mice (Walton et al. 2009). The inhibitors PF670462, IC261, D4476, and longdaysin are shown to lengthen circadian period considerably by inhibiting CK1δ-dependent phosphorylation and subsequent degradation of PER proteins in a dose-dependent manner (Walton et al. 2009; Isojima et al. 2009; Hirota et al. 2010; Long et al. 2012; Rena et al. 2004; Mashhoon et al. 2000). Along with its role in period lengthening, PF670462 has been shown to reduce relapse-like drinking behavior in mice in a dose-dependent manner, suggesting a role of CK1δ/ε in addictive behavior (Perreau-Lenz et al. 2012). CK01, an inhibitor of CK1, also has period-lengthening effects and, interestingly, is found to entrain the rhythms in arrhythmic animals (Arey and McClung 2012). Additionally, chronic administration of CK01 was demonstrated to reduce anxiety-related behavior in clock mutant ($Clock^{\Delta 19}$) mice that display behavioral characteristics similar to human mania (Arey and McClung 2012). These studies provide evidence that targeting CK kinases is a viable method to study and alter circadian period and circadian-related disorders.

While kinases are very popular targets for drug designing, the ability to develop a highly specific compound targeting a particular kinase is very challenging due to the high degree of conservation between kinase family members. Most of the kinase inhibitors appear to target multiple enzymes. The kinase inhibitor PF670462 has an IC_{50} of 8–14 nM for CK1δ/ε but is also demonstrated to have significant activity toward EGFR, p38 kinase, PKA alpha, MAP4K4, and CK1α (Walton et al. 2009; Long et al. 2012). Structural biology approaches have been taken tounderstand these off-target effects and optimize the compounds for enhanced specificity. A study revealed the structural basis of CK1δ and ε interaction with PF670462 and suggested ways to optimize CK1δ/ε inhibitors (Long et al. 2012). Another study used structure-based drug design to develop selective inhibitors of CK1δ. The difference in the hinge region of CK1δ and p38 was identified and this difference

was used in designing CK inhibitors with more selective pharmacological targeting (Mente et al. 2013). These compounds will allow the study of isoform-specific effects of CK1δ kinase on circadian clock and cellular processes without affecting paralogous enzymes.

The important effects of these kinases on period length and the evidence showing the ability of kinase inhibitors to alter period in animal models suggest that CK1δ/ε kinase inhibitors are attractive targets for treatment of sleep disorders and jet lag. However, the promiscuity of some of these drugs in hitting multiple unwanted targets warrants the need to employ structure-based drug design and identify ways to design highly specific kinase inhibitors.

12.3.7 Phosphatases

Protein posttranslational modification by protein serine/threonine phosphorylation appears to be involved in a plethora of pathways in the eukaryotic cell. This posttranslational modification may change the behavior of a protein, its binding ability, its cellular localization, and its stability. This function is balanced by phosphatase activity. There exist only a few known phosphoserine/threonine phosphatases (PTSPs) to counter the activity of serine/threonine kinases, which account for about two-thirds of the ~500 kinases encoded in the human genome. In fact, there have been 150 identified protein phosphatases out of which only one-third are PSTPs. PSTPs generally act as hetero-oligomeric complexes. PSTPs compensate for their low numbers by combining catalytic subunits with different regulatory subunits, thus creating a variety of phosphatase holoenzymes. Currently, the catalytic subunit of serine/threonine phosphatases is not completely yet understood; thus, the number of these phosphatases may increase in future studies. Since more than 98 % of protein phosphorylation occurs on serine/threonine residues, the identification of compounds that affect specific serine/threonine phosphatases seems especially promising for drug development.

The subfamily of the phosphoprotein phosphatases (PPPs)—PP1, PP2A, PP4, and PP5—family seem to function to counteract CK1δ/ε phosphorylating activity and thus play an important role in circadian function (Moorhead et al. 2009; Shi 2009). PPPs dephosphorylate PER2 sites that are phosphorylated and destabilized by CK1δ/ε (Yang et al. 2004; Gallego et al. 2006; Fang et al. 2007). Many clock molecules are rhythmically phosphorylated, which is achieved by the tightly regulated action of kinases and phosphatases. PER2 is the most studied component on the clock in terms of its cycling phosphorylation control. It contains 247 serine or threonine residues that are potential phosphorylation targets (Lee et al. 2001).

The ubiquitous PP1 interacts with over 50 described regulatory target subunits via its promiscuous catalytic domain. This subfamily is characterized by its high activity and low specificity. Dephosphorylation activity occurs via the formation of a phospho-monoester bond, which shows high stability at neutral pH. In this stable environment, the PP1 catalytic subunit accelerates the rate of hydrolysis by 10^{21},

making PP1 a highly efficient enzyme (Lad et al. 2003). There are four known catalytic subunit isoforms of PP1 (α, β, γ1, and γ2), which are encoded by three different genes (Ceulemans and Bollen 2004). The structure of the catalytic subunit is highly conserved, with variability in the C-terminus. The C-terminal domain may be responsible for ligand as well as target binding (Watanabe et al. 2001; Terrak et al. 2004). Most regulatory subunits contain a specific RVxF site, which is necessary for binding the catalytic subunit (Wakula et al. 2003).

Crystallographic studies of heterodimer catalytic subunit PP1γ and RVxF targeting subunit MYPT1 (myosin phosphatase target subunit 1), which target and dephosphorylate myosin light chain (MLC), revealed how the targeting subunit regulates the catalytic subunit. The exposed catalytic subunit has an unprotected pocket that allows binding of many substrates. Upon interaction with PP1δ C-terminus, the conserved RVxF subunit, MYPT1, causes major pocket conformational rearrangements that make the catalytic subunit specific for the target, myosin light chain (MLC) (Wakula et al. 2003).

In the bread mold, *N. crassa*, PP1 has been shown to dephosphorylate FRQ (the *Neurospora* homologue to PER2, nPer2) in vitro. Thus, the degradation rate of circadian transcription inhibitor FRQ is regulated by PP1. Finally, the mutant catalytic subunit of *pp1* is associated with advanced phase shift and shorter period (Yang et al. 2004). In the fruit fly, *D. melanogaster*, destabilization of TIM (dPer2) via PP1 has been demonstrated using PP1 inhibitors (Fang et al. 2007). In the mammalian system, PP1 has been shown to directly interact with PER2 by co-immunoprecipitation assays in HEK cells (Gallego et al. 2006). The use of a dominant negative catalytic PP1 subunit causes accelerated degradation of PER2. Moreover, PP1 was shown to dephosphorylate CK1ε-mediated phosphorylated sites (Gallego et al. 2006). Inhibitors of this phosphatase may be considered as therapeutic targets to manipulate the clock.

Some PP1 inhibitors comprise Inhibitor-1, Inhibitor-2, microcystin (MC), okadaic acid (OA), DARPP-32, NIPP-1, and calyculin A (Cal A) (Honkanen and Golden 2002). Nuclear inhibitor of PP1 (NIPP1) is very potent with an IC_{50} value of less than 1 pM. It shows high specificity for PP1, as it does not affect PP2. Studies in *Drosophila* showed that NIPP1-overexpressing flies increased period lengthening in TIM^{UL} background.

PP2A is characterized by high activity and low specificity both in vitro and in vivo. It works by forming hetero-oligomers. In fact, heterotrimer formation seems to be necessary for PP2A stability. The heterotrimeric architecture is composed of scaffold, catalytic, and variable regulatory subunits (Li et al. 2002; Silverstein et al. 2002). There are two identified isoforms of the catalytic subunit (a and b), generated from two separate genes. It has been associated with two regulatory subunits, PPP2R1-3 and PPP2R5. Although its function has not been described in the mammalian system, its role has been extensively studied in *D. melanogaster* and *Neurospora*. In *Neurospora*, PP2A dephosphorylates FRQ, and decreased *Frq* mRNA and protein levels were detected in *rgb-1* (regulatory PP2 subunit) mutants in vivo, resulting in long periods and decreased amplitude of the rhythm (Yang et al. 2004). Moreover, rhythmicity of circadian behavior has been

compromised by either PP2A inhibition or overexpression (Schafmeier et al. 2005). In *Drosophila*, PP2A has been found to stabilize PER and CLOCK (Kim and Edery 2006). Therefore, it is involved in increase of nuclear concentration of PER/TIM complex. PP2 inhibitors include fostriecin, OA, MC, Cal A, and nodularin (Honkanen and Golden 2002).

PP5 is ubiquitously expressed and encoded by a single gene. Its subcellular-targeting and regulatory domains are contained within a single peptide chain, unlike PP2A and PP1. However, its catalytic activity resembles that of PP2B (Swingle et al. 2004) and PP1 and shares about 40 % sequence identity with other PPPs (Swingle et al. 2004). It has, as a unique feature, the presence of a tetratricopeptide repeat (TPR) motif in the N-terminal domain plus a C-terminal domain. The TPR region plays a crucial role in the interaction of PP5 with other proteins (Honkanen and Golden 2002), and the N-terminal domain possesses autoinhibitory properties and plays a role in targeting the protein to the nucleus (Sinclair et al. 1999; Borthwick et al. 2001; Kang et al. 2001). In a mammalian circadian system, PP5 has been shown to interact with CK1ε, CRY1, and CRY2 in co-immunoprecipitation assays (Partch et al. 2006). PP5 binds to CK1ε through its C-terminal catalytic domain; it dephosphorylates CK1ε's autoinhibitory terminal tail, which results in increased CK1ε activity followed by destabilization of PER 2 phosphorylation and subsequent degradation. CRY2 also interacts with PP5, but via the TPR domain. This suggests the possibility of formation of CRY-PP5-CK1ε complexes. CRY-bound PP5 complexes result in a decrease of PP5-mediated activation of CK1ε (Partch et al. 2006). PP5's known inhibitors are okadaic acid, MC, Cal A, and nodularin (Lubert et al. 2001).

PP4 has also been implicated in regulation of the circadian rhythm based on data obtained in *Neurospora*. Loss of PP4 results in decreased period length and a low rhythm amplitude (Cha et al. 2008). Its described inhibitors are Fostriecin, okadaic acid, MC, Cal A, and nodularin (Honkanen and Golden 2002).

Discovery of natural products and toxins that inhibit PSTPs has allowed for characterization of the role these enzymes play in various cellular functions. The chemical structure of such compounds ranges from lipids to cyclic peptides. Still, despite the wide structural variability, they target the same phosphatases (Cohen et al. 1990). The most toxic inhibitors (MC-LR, Cal-A, and nodularin) are the most potent inhibitors (at μM concentrations) of PP1, PP2A, PP4, and PP5. Okadaic acid and calyculin A are cell-permeable tumorigenic compounds (Fujiki and Suganuma 1993). Other inhibitors may accumulate in the liver, causing hepatic tumors (Nishiwaki-Matsushima et al. 1991). These toxins have been implicated in cell growth inhibition, highlighting the importance of target phosphatases in different stages of cell division. Interestingly, okadaic acid has also been shown to decrease methylation of PP2A catalytic subunits (Li and Damuni 1994).

Inhibitor-1 (I-1) and Inhibitor-2 (I-2) are proteins and were first isolated from rabbit skeletal muscle (Huang and Glinsmann 1976a, b). I-1 phosphatase inhibitory activity is dependent on cAMP-dependent protein kinase A (PKA) phosphorylation, whereas I-2 is constitutively active. I-1 is expressed in many mammalian tissues (Elbrecht et al. 1990), while structurally homologous protein DARPP-32 expresses

in the central nervous system (Walaas and Greengard 1991). Both I-1 and DARPP-32 inhibit PP1 in response to hormones and neurotransmitters that activate cAMP (Cohen 1989; Mumby and Walter 1991; Shenolikar and Nairn 1991). Sequence homology between I-1 and DARPP-32 revealed a domain sufficient to target PP1 inhibition. Such domain served as a starting point to synthesize peptides to target PP1 (Hemmings et al. 1990). It also permitted the identification of an isoleucine at position 9 that determined PP1 inhibition. Using surface plasmon resonance spectroscopy, it was determined that the isoleucine binds near PP1 catalytic domain, inhibiting phosphatase activity (Desdouits et al. 1995). I-2, injected intracellularly, inhibits PP1 activity (Foulkes and Maller 1982). I-2 forms a complex with PP1 catalytic subunit, rendering it inactive. Some evidence suggests that I-2 may act as a PP1 chaperone (Alessi et al. 1993). Inhibitor-1 of PP2A ($I\text{-}1^{PP2A}$) and inhibitor-1 of PP2A ($I\text{-}2^{PP2A}$) inhibit the phosphatase with an IC_{50} of 30 and 25 nM, respectively (Li et al. 1995). Another protein inhibitor, NIPP-1, is the most potent phosphatase inhibitor to be characterized (Beullens et al. 1992). However, its efficacy is decreased upon phosphorylation by PKA and casein kinase II (Van Eynde et al. 1994; Beullens et al. 1992).

Microcystins are a family of nearly 50 heptapeptides isolated from cyanobacteria, *Microcystis aeruginosa*. The most common member of the family, microcystin-LR (variable amino acids leucine and arginine), interacts with three regions of PP1. Its structure remains unchanged when bound to PP1, while PP1 undergoes a conformational change (particularly in the β12/β13 loop) to allow optimal binding with the inhibitor (Bagu et al. 1995; Barford 1996). Their tight interaction with the phosphatases results in IC_{50} values that range in the nM level.

Okadaic acid (OA) is a polyether fatty acid that was first isolated from the marine black sponges *Halichondria okadai* and *Halichondria melanodocia*. In humans, it is associated with diarrheic shellfish poisoning due to its toxicity (Pistocchi et al. 2012). Although mainly considered an enterotoxin (Edebo et al. 1988), OA is associated with systemic immunotoxicity in mice, as well as hepatotoxicity, skin irritation, organ morpho-functional changes, and tumorigenesis at sublethal doses (Franchini et al. 2010; Franchinia et al. 2005; Sontag and Sontag 2006; Dounay and Forsyth 2002). Its toxicity is attributed to its activity as a phosphatase inhibitor. OA is a potent inhibitor of PP2A and PP1, with IC_{50} values of 0.2 μM and 20 μM, respectively (Cohen 1991). However, it also inhibits PP4 and PP5. After OA total synthesis, its structure has been determined by X-ray crystallography (Dounay and Forsyth 2002), which led to considerable structure-based studies directed toward the development of potential therapeutic targeting PSTPs with lower toxicity. Its promiscuity with other phosphatases as well as toxicity makes it difficult to determine its direct path.

Two additional natural products are fostriecin and nodularin. Fostriecin, a phosphate monoester antibiotic produced by *Streptomyces pulveraceus*, has been shown to possess strong antitumor properties via selective inhibition of PP2A and PP4 resulting in interference with the mitotic entry checkpoint. Synthetic analogues have been generated containing more desirable structural features from the fostriecin scaffold (Lewy et al. 2002). Fostriecin enters the cell via a

transporter—the reduced folate carrier system (Fry et al. 1984). Nodularin is a cyclic peptide produced by *Nodularia spumigena*, a cyanobacterium.

About 10 variants of nodularin have been identified to date. Nodularins are cyclic peptides and do not readily enter most cell types. Cal A readily enters through cell membranes, but is insoluble in aqueous solutions. The concentration most commonly employed (50–100 nM) in cell culture would result in cell death in vivo once the intracellular concentration reaches 10 nM (Swingle et al. 2007). Many of these small molecules are very potent phosphatase inhibitors. Most, however, exhibit promiscuity, which makes it challenging to determine the actions of broad classes of phosphatases.

12.4 Conclusion

As the components of the molecular clock have been identified, there has been increasing interest in developing chemical tools to specifically modulate the activity of these proteins to determine the effect that they may have on the circadian rhythm as well as physiological function and their potential to treat human disease. Over the past several years, a number of small molecule drugs have been designed that target components of the clock such as the CKs, REV-ERBs, RORs, and CRYs. Many studies have demonstrated that there may indeed be utility for these classes of drugs to treat metabolic disorders and behavioral disorders. As these clock components have been validated as drug targets, we expect considerably more focus on the circadian clock as a point of pharmacological modulation in the future development of human therapeutics.

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