METABOLISM AND THE CIRCADIAN CLOCK CONVERGE

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Eckel-Mahan K, Sassone-Corsi P. Metabolism and the Circadian Clock Converge. Physiol Rev 93: 107–135, 2013; doi:10.1152/physrev.00016.2012.—Circadian rhythms occur in almost all species and control vital aspects of our physiology, from sleeping and waking to neurotransmitter secretion and cellular metabolism. Epidemiological studies from recent decades have supported a unique role for circadian rhythm in metabolism. As evidenced by individuals working night or rotating shifts, but also by rodent models of circadian arrhythmia, disruption of the circadian cycle is strongly associated with metabolic imbalance. Some genetically engineered mouse models of circadian rhythmicity are obese and show hallmark signs of the metabolic syndrome. Whether these phenotypes are due to the loss of distinct circadian clock genes within a specific tissue versus the disruption of rhythmic physiological activities (such as eating and sleeping) remains a cynosure within the fields of chronobiology and metabolism. Becoming more apparent is that from metabolites to transcription factors, the circadian clock interfaces with metabolism in numerous ways that are essential for maintaining metabolic homeostasis.

A. A Brief Overview of Circadian Physiology

The word circadian derives from the Latin circa (around) and dies (day). Oscillations of ~24-h periodicity are referred to as circadian (FIGURE 1A). The molecular and physiological oscillations discussed here may vary in amplitude and even phase; however, in common is their ~24-h periodicity (or tau, \( \tau \)), which temporally follows the earth’s rotation around its axis. Zeitgebers (the German word for “time givers”) are signals that help synchronize the body’s circadian clock with the environment (TABLE 1). In mammals, light is one such zeitgeber and functions as such by activating a small region of the hypothalamus located just above the optic chiasm. Lesion studies identified this region, the suprachiasmatic nucleus (SCN), as being light responsive as lesions within this area abolished rhythmic circadian behavior in both locomotion and food consumption (224). The SCN is located in the anterior hypothalamus and is comprised of a meager 20,000 neurons, which receive photic information from the environment via neurons transcending from the retina via the retino-hypothalamic tract. The circadian system is organized hierarchically, meaning that while molecular oscillations occur in most cells and tissues of the body, the SCN functions as the master regulator, synchronizing the phase of other oscillating tissues (95, 204). Its superiority in this sense has been demonstrated in grafting experiments; SCN grafts from wild-type (WT) mice into a genetically arrhythmic mouse can restore normal circadian rhythmicity (227). Examples of SCN-directed circadian rhythms include feeding, the sleep-wake cycle, glucose metabolism, insulin secretion, and even learning and memory (95). Various techniques have been employed to measure and depict the properties of these rhythms at the systems level.
rhythms including the simple actogram. Actograms graphically portray these rhythms, plotting activity in such a way that both the phase and period of the oscillation can be obtained. **FIGURE 1B** shows the actogram of a nocturnal animal’s activity during the 24-h day, with dark shading representing their activity (in this case, wheel running) and blank areas reflecting the animal’s rest or sleeping period.

Central to the molecular rhythmicity of SCN neurons as well as other oscillating cells are transcription factors that drive expression of their own negative regulators (204). This property of the clock results in a negative transcriptional and translational feedback loop that perpetuates oscillations in gene expression that occur every 24 h (**FIGURE 1C**). This program is highly conserved across species. **FIG-U-
### Table 1. Definitions for common circadian terminology and the core clock components of the mammalian circadian clock

<table>
<thead>
<tr>
<th>Term</th>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circadian</td>
<td></td>
<td>Refers to the ∼24-h nature of an event. Circadian is derived from the Latin roots “circam” (about) and “diem” (day).</td>
</tr>
<tr>
<td>Entrainment</td>
<td></td>
<td>Adaptation over time to an imposed cue such as light or food.</td>
</tr>
<tr>
<td>Zeitgeber</td>
<td>ZT</td>
<td>German word meaning “time giver”. A cue [such as light or food] that entrains the circadian clock. ZT refers to “zeitgeber time.”</td>
</tr>
<tr>
<td>Suprachiasmatic nucleus</td>
<td>SCN</td>
<td>A small region of the anterior hypothalamus consisting of bilateral nuclei that coordinately synchronize rhythmicity within other tissues.</td>
</tr>
<tr>
<td>Circadian clock-controlled gene</td>
<td>CCG</td>
<td>A gene expressed in a circadian-dependent manner, usually under the control of a promoter that contains an E-box, D-box, or RRE.</td>
</tr>
<tr>
<td>Brain and muscle Arnt-like protein-1</td>
<td>BMAL1</td>
<td>The mammalian bHLH-PAS transcription factor that dimerizes with CLOCK and NPAS2 to activate gene transcription.</td>
</tr>
<tr>
<td>Circadian locomotor output cycles kaput</td>
<td>CLOCK</td>
<td>The mammalian bHLH-PAS transcription factor that dimerizes with BMAL1 to activate promoters that contain E-boxes (CAGGTG).</td>
</tr>
<tr>
<td>Cryptochrome proteins</td>
<td>CRY</td>
<td>Transcriptional repressors that dimerize with PER to inhibit CLOCK:BMAL1-mediated gene transcription. In plants and invertebrates, these function as light-responsive flavoproteins.</td>
</tr>
<tr>
<td>Neuronal PAS domain protein 2</td>
<td>NPAS2</td>
<td>A transcription factor similar to CLOCK and highly expressed in the forebrain. NPAS2 dimerizes with BMAL1 to activate gene transcription.</td>
</tr>
<tr>
<td>Period homolog proteins</td>
<td>PER</td>
<td>PAS domain containing proteins which dimerize with TIM (in Drosophila) or CRY (in mammals) to inhibit CLOCK:BMAL1-induced gene transcription. The Drosophila Period gene was the first circadian gene discovered.</td>
</tr>
</tbody>
</table>

**URE 1D** demonstrates the corresponding positive- and negative-feedback proteins of this molecular system across several species. With the exception of the cyanobacteria circadian clock, the proteins in this table participate in the negative transcriptional translational feedback loop described. In mammals, two basic helix-loop-helix (bHLH) transcription factors, CLOCK and BMAL1 (also known as MOP3 or ARNT1), heterodimerize and subsequently bind to conserved E-box sequences in target promoter genes. In this way, they drive the rhythmic expression of mammalian Period (Per1, Per2, and Per3) and Cryptochrome (Cry1 and Cry2) genes (FIGURE 2). PER and CRY proteins form a complex that translocates back to the nucleus to inhibit CLOCK:BMAL1-mediated gene expression. This deceivingly simple transcriptional feedback loop is regulated by highly complex mechanisms (such as posttranslational modification of circadian proteins and additional interlocking feedback loops of gene transcription) that mandate fine-tuning of the clock and yet provide for plasticity by which it can adjust to changes in the environment (21, 38). While the clock genes are necessary for circadian physiology, a number of clock controlled genes (CCGs) also contribute. These are genes that are regulated by the circadian clock (and therefore oscillate with 24-h periodicity) but are not necessarily conserved across tissues.

While the view that this transcriptional and translational feedback loop is essential for timekeeping in cells has held preeminence, recent studies may revise the classical view of how rhythmicity is maintained within the cell. New data reveal that circadian rhythms can persist even in the absence of transcription. Specifically, the posttranslational modification of some proteins can occur in a transcription-independent manner (167, 168). This has been demonstrated by rhythmicity in the oxidation of peroxiredoxin proteins, for example, which is temperature compensated and entrained by zeitgebers, two fundamental qualities of circadian rhythms. While these transcription-independent oscillations occur, they do appear to exist in collaboration with the classical transcriptional loops described, as nucleated mouse embryonic fibroblasts from circadian arrhythmic animals show altered but present rhythmic peroxiredoxin oxidation. Thus there appears to be direct interaction between nuclear and cytoplasmic circadian rhythms when there is a nucleus present (168). Interestingly, the circadian oxidation-reduction cycles of peroxiredoxin are very highly conserved, even more so than the conservation that exists for the circadian clock components of **FIGURE 1D** (62). The idea that oscillations can persist in the absence of a nucleus is important because it means that there are likely numerous zeitgebers for the clock that are yet unidentified as such. In fact, the metabolome in its entirety may need to be addressed as possible zeitgebers and may feed into the clock system in specific ways, affecting phase, amplitude, or period of existing transcriptionally dependent oscillations. The concept of metabolites as critical modulators of the circadian clock will be further addressed in section IVB.

Circadian rhythmicity can be seen in many physiological processes. Examples include body temperature, activity, sleep, metabolism, heart rate, blood pressure, and hormone and neurotransmitter secretion (95). This being the case, it is perhaps not surprising that many cellular processes, including gene expression, oscillate within the cell.
mately 10% of gene transcripts oscillate in the cell; however, a much larger percent of the proteome oscillates in either expression or activity (25, 57, 149, 180, 190). The large number of transcripts that oscillate in the cell depends in part on circadian changes in chromatin remodeling, perpetuated by rhythmic alterations in histone modifications such as phosphorylation or acetylation (161). Histone acetyltransferase and deacetylase activity at oscillating genes serves as a preamble for much of the rhythmic gene expression in vivo. Some of the chromatin modifying enzymes responsible for this activity may associate directly with circadian clock proteins (6, 162). CLOCK itself contains histone acetyltransferase activity which contributes to the rhythmic acetylation of K14 of histone H3 and K537 of its binding partner, BMAL1 (99). Recently, metabolites that fuel these rhythmic events have been observed to oscillate, sometimes producing rhythmic enzymatic activity even when the expression of a substrate’s enzyme doesn’t oscillate (163, 189). As more metabolites that contribute to clock maintenance are being identified, it is becoming evident that metabolism and circadian rhythmicity are intertwined molecularly and that one process cannot be adequately studied in isolation of the other. While much remains to be revealed in terms of their interactions, how oscillators in various compartments, from cells to tissues, interact to control metabolic physiology is beginning to be understood more fully.

B. Metabolic Homeostasis

Western (and what we generally consider as “westernized”) societies are experiencing a dramatic rise in metabolic disorders (14, 32, 61, 222). This rise is not limited to the adult population, as evidenced by an increase in overweight children and adolescents (222). The production of high-energy foods that are nutritionally wanting as well as the decrease in energy expenditure often associated with jobs that allow a sedentary life-style have certainly contributed to the occurrence of metabolic disorders. Genetics also contributes to alterations in metabolic function (88). In mammals, both environmentally induced circadian disruption as well as genetic aberrations in circadian clock machinery can lead to metabolic disorders (reviewed in Ref. 75). Why circadian disruption has this effect on metabolic homeostasis appears to be complex.

**FIGURE 2.** Additional inputs to the core circadian loop. The basic positive and negative transcriptional feedback loop of the circadian clock is elaborate, with external loops and posttranslational modifications, such as phosphorylation contributing to maintenance of the core oscillatory players. 5′ AMP-activated protein kinase (AMPK) and casein kinase I epsilon (CKIε) contribute to phosphorylation and degradation of the CRY and PER proteins, respectively, thus regulating the negative-feedback potential of these proteins on the CLOCK:BMAL1 complex. The transcription of Bmal1 is negatively regulated by one of its own gene targets, Rev-erbx, the process of which controls the amount of BMAL1 protein available for CLOCK binding. Retinoic acid receptor-related orphan receptor alpha (RORα) exerts opposite effects to REV-ERBα on the Bmal1 promoter.
Energy balance is when energy (food) intake is equal to energy expenditure. Major sources of metabolic fuels include glucose, fatty acids, and ketone bodies. If not consumed, metabolic fuels are converted into metabolic stores including liver glycogen, muscle protein, and the triglycerides found in adipose tissue. Energy expenditure is a composite of three components: basal metabolic rate (energy to keep the nervous system working and the vital organs functioning properly), thermogenesis (the energy required for the absorption and storage of food), and physical activity (the most variable). Whether one is a 1.58 m, 86.2 kg individual or a 1.85 m, 72.6 kg individual, the body is remarkable in its ability to maintain energy equilibrium.

While genetics indubitably contributes to the body’s ability to maintain energy balance (61, 88, 98), numerous studies have revealed that environmental cues including those affecting one’s circadian rhythms also play essential roles in metabolic homeostasis (71, 97). With modern technology (which makes provisions such as exogenous lighting throughout earth’s 24-h day), night shift and rotating shift work have become increasingly common. Such alterations beyond the conventional workday alter not only temporal aspects of work and social activities but also physiological and molecular rhythms in the body. Compelling evidence that circadian rhythm disruptions contribute to metabolic disorders has been observed in experiments centered on night shift and rotating shift workers. For example, an association between circadian disturbance and cardiovascular disease, increased body mass, and elevated plasma glucose and lipid levels has been observed in humans subjected to nighttime shift work (118, 120, 182, 234). Rodent studies also support this link as simply altering their normal light-dark cycle to one in which dim light replaces the normal dark period causes changes in metabolism that are observed on a physiological level (70). The integration of these processes at the cellular and systems level will be discussed further in later sections.

Rhythms in energy intake must coincide with endogenous fluctuations in gene expression for metabolic homeostasis at the cellular level to occur. At the cellular level, energy from our diet is used to generate macromolecules such as proteins, DNA, membrane components, and polysaccharides. As energy intake is a circadian activity, it drives fluctuations in the rate of these activities in different tissues. Furthermore, many of the oscillating gene transcripts in a given tissue are tissue specific or at least oscillate in a tissue-specific fashion (226). Tissues thereby meet their metabolic demands using regulatory molecules that are temporally or spatially distinct from other tissues. For example, many hormones that control food intake including insulin, glucagon, peptide YY, GLP-1, corticosterone, leptin, and ghrelin depend on energy intake or oscillate in a circadian manner, and are largely secreted in a tissue-specific fashion (reviewed in Ref. 75). The contrasting profile of nuclear receptor expression across different tissues also emphasizes the tissue-specific nature of metabolic gene expression. Numerous transcription factors, including metabolic nuclear receptors, display 24-h periodicity (250). These include receptors such as FXR, LXR, HNF-4α, PPARα, PPARγ, NUR77, and many others (www.nursa.org/10.1621/datasets.02001) that are more highly expressed in some metabolic tissues over others.

In summary, evidence that circadian and metabolic processes interact at the cellular levels is gaining strength and may help provide molecular explanations for the growing number of metabolic disorders associated with circadian disruption. Studies addressing this link at both the system and cellular levels are being performed and are illuminating some surprising effects of the clock on physiology.

II. SYSTEMS HOMEOSTASIS AND CIRCADIAN RHYTHMICITY OF PROCESSES INVOLVED IN ENERGY BALANCE

A. Feeding Is a Circadian Rhythm

The circadian regulation of energy intake is consistent across mammals. Mammals tend to consume the vast majority of food and water during their waking period. This occurs during the day for diurnal mammals and during the night for nocturnal animals and coincides with food-seeking activity. In mammals, feeding is under homeostatic control and involves humoral factors acting on hypothalamic neurons that ultimately control the urge to feed or not to feed. This is accomplished via endocrine molecules including leptin, ghrelin, and peptide YY acting on neurons within the hypothalamic arcuate nucleus (63). Neurons of the paraventricular nucleus both sense and integrate the orexigenic and anorexigenic signals that result from these hormones (reviewed in Ref. 181). These and other biological signals that control energy intake will be discussed in more detail in section IIIA2. Early lesion studies identified the SCN’s contribution to circadian rhythmicity in eating and drinking as ablation of the SCN destroys rhythmicity in both eating and drinking (224). While it has been argued that early lesion studies may have involved tissue injury that extended beyond the SCN to other hypothalamic circuits, other models of circadian arrhythmicity also appear to be arrhythmic in energy intake when fed ad libitum (237). To some extent then, feeding is controlled directly or indirectly both by circadian and homeostatic processes.

Feeding that follows a typical pattern of daytime eating for diurnal organisms or nighttime eating for nocturnal organisms seems to be important for metabolic homeostasis. One recent study addressing the role of the circadian clock in metabolic function showed a somewhat surprising result: simply replacing the dark period of a rodent’s circadian cycle with very dim light produced a pronounced increase in
the rodents’ body mass (70). Using a 16-h light/8-h dark cycle paradigm for control animals, the experimenters exposed some mice to a 16-h light/8-h dim light or a constitutively light paradigm. Interestingly, exposure to dim light at night caused increased energy intake during the daytime hours compared with animals in normal conditions, although total energy intake was unchanged between the two groups. An increase in body mass was detected in both experimental groups after only 1 wk of exposure to the new lighting paradigm, and this increase continued throughout the 8-wk program in the new lighting conditions. Epididymal fat pads were enlarged in experimental groups, indicating that changing the lighting conditions had an effect on white adipose deposition. As locomotion, corticosterone levels, and total 24-h energy intake were all unaltered in one or both of the experimental groups, only the increased percentage of energy intake that occurred during the day could account for the increased body mass observed in these rodents. When dim light-exposed animals were restricted to only nighttime feeding, the body mass alterations were prevented. While this study presents evidence that the normal circadian rhythmicity in food intake is important for metabolic homeostasis in rodents, the results support human studies in which night-eating syndrome (NES) is associated with obesity and circadian misalignment (170, 202, 231).

NES and sleep-related eating disorder (SRED) are two related eating disorders that include conscious or unconscious food consumption at night. They are typically associated with morning anorexia and evening hyperphagia as well as perpetual awakenings during the night that often involve eating (reviewed in Ref. 169). NES-inflicted individuals may wake from one to four times at night, and 74% of all awakenings are associated with food consumption (170). NES patients show a delayed melatonin rhythm and a phase advance of orexigenic ghrelin (which is released predominantly by the gastrointestinal tract) rhythms. The amplitude of ghrelin rhythms is also decreased in NES individuals (81). While plasma glucose levels are antiphase to control human subjects, insulin levels are phase delayed and are also considerably reduced in amplitude in NES-afflicted subjects. As NES reflects a phase delay in the acquisition of food, potential circadian mechanisms underlying the disease are under consideration. Sertaline, a selective serotonin reuptake inhibitor (SSRI), has been shown to reduce the amount of nighttime calories consumed in NES-afflicted individuals and may do so by altering rhythms at the level of the SCN (171). It is still unclear whether NES individuals represent a situation in which uncoupling of peripheral oscillators with the central clock occurs or whether individual peripheral oscillators are functioning out of phase with each other independent of central clock coherence. NES-afflicted individuals, however, support other evidence that implicates clock disturbances with metabolic disorder.

B. Food as a Zeitgeber, a Metabolic Circadian Cue

While originally thought to be limited to the brain, the occurrence of circadian rhythms has been noted throughout tissues of the body. In fact, most tissues studied to date show robust oscillations in gene expression (254). There are few known zeitgebers, however, that can entrain the circadian clock in vivo. Those identified include light and, more recently, food. As a light pulse during the subjective night can phase advance or delay the SCN circadian clock, food can function as a potent zeitgeber for peripheral tissues, underscoring the important relationship between circadian and metabolic processes. The circadian rhythm of the SCN, which responds robustly to light, is largely unaffected by changes in feeding patterns, while oscillations within some peripheral tissues, such as the liver, appear to be dependent on communication with a rhythmic brain but principally on the feeding cycle. For diurnal animals, like humans, feeding occurs during the day, whereas for nocturnal organisms, food intake takes place predominantly at night, when they are awake and active. Feeding and circadian rhythms in gene expression are so tightly linked that, when food is restricted to a precise time of the day, the expression of a large number of hepatic genes is altered, with a new rhythm that follows that of the feeding cycle (37, 225). One study demonstrating the zeitgeber property of food involved mice that were restricted to 4 h of feeding during their rest period. As early as 2 days after restricted feeding, the liver showed a 10-h phase change in circadian rhythmicity. Analysis of several tissues from animals that were fasted following a week of restricted feeding showed that not only the liver but also the lung had been entraining to the new restricted feeding schedule. Conversely, in a liver which lacks normal circadian rhythmicity, the majority of hepatic gene expression rhythms can be restored by exposure to a temporally restricted feeding schedule (237). Technically, food can entrain both the periphery and the brain, as a freely running animal that is exposed to a restricted feeding schedule can show signs of SCN entrainment (as measured by wheel running, for example) that may be caused directly or indirectly by food and the anticipatory activity involved in its administration (reviewed in Ref. 156). The restriction of feeding to a few hours during an animal’s normal resting period results in food anticipatory activity (FAA), in which locomotion patterns deviate from normal circadian cycles (64). Such enhanced activity during the rest period serves as a preamble to the feeding event, indicative of the animal’s anticipation of food consumption. While still under investigation, a functional clock in the dorsomedial hypothalamus (DMH) has been implicated in FAA (77). Bmal1 knockout animals and Clock mutant animals, both of which show complete arrhythmia, however, can still show FAA under some experimental conditions (183, 185), so it is unclear what role the circadian clock might play in the central nervous system for FAA to occur. Entrainment to food can also occur in rodents with SCN lesions (127, 223),
indicating that the neuronal locations governing FAA are at least partially distinct from those which participate in light entrainment. Interestingly, ghrelin seems to stimulate the appetitive and consummatory aspects of food intake during feeding restriction, and ghrelin receptor knockout animals show a reduction in FAA (141). While the arcuate nucleus is one of several ghrelin-expressing regions of the brain that could account for the change in FAA in ghrelin receptor knockout animals, other molecules in satiety centers also contribute to this process. The melanocortin 3 receptor, for example, is highly expressed in food-responsive regions of the hypothalamus and responds to anorexigenic hormones so as to lower energy intake and increase energy expenditure. Melanocortin 3-receptor knockout mice have increased adiposity (27) and, interestingly, melanocortin 3 knockout mice are immune to FAA (16, 228). Knockout of the melanocortin 3 receptor in mice demonstrates its importance for the circadian timing of food consumption but also for entrainment of anticipatory behavior to feeding time.

In summary, feeding is a circadian event. However, it serves not only as an output of the clock but also as a clock input mechanism, particularly for peripheral tissues. Because peripheral tissues communicate back to the brain via ghrelin, leptin, glucose, insulin, etc., circadian feeding contributes to an intertwining of the clock and metabolism that appears to be crucial for metabolic homeostasis.

C. Circadian Rhythmicity of Energy Expenditure

Energy expenditure involves the maintenance of one’s basal metabolic rate, the maintenance of resting metabolic rate, physical exercise, and the maintenance of core body temperature. The vast majority of energy expended in an organism goes towards maintaining one’s basal metabolic rate, and this directly affects body temperature. Core body temperature oscillates in a circadian fashion with a peak that occurs during the early evening in humans and a trough during the early morning hours. Human subjects isolated in free running conditions (i.e., in the absence of zeitgebers such as light) still show oscillations in core body temperature and sleep/wake cycles (259). Heart rate follows the temporal profile of body temperature, with beats per minute reaching a nadir around ZT3 in human subjects. Interestingly, thermoregulatory changes appear to be tied into the sleep/wake cycle and are important for the circadian modulation of sleepiness and propensity to sleep (126). A fraction of spent energy is in the form of physical exercise, and this type of energy expenditure is highly variable across the population. Some human performance measures including muscle strength, anaerobic power output, and joint flexibility follow the circadian pattern of core body temperature (reviewed in Ref. 192). Therefore, studies have addressed whether sports performance, for example, or exercise is optimal at specific zeitgeber times. An interesting concept is that rigorous exercise might be part of the reciprocal interplay between the circadian clock and metabolism. Recent attention has been paid to the idea that exercise might actually function as a zeitgeber for the body. This promises to be an intriguing area for future study.

D. Malfunctioning Clocks and Metabolic Disease

Recent data from night shift workers and individuals with sleep disorders provide evidence that metabolism and circadian rhythms are tightly linked in vivo. The implications of this are probably widespread. With the use of the definition that shift work is that which occurs during nontraditional working hours (usually from 10 p.m. to 6 a.m.), almost 20% of workers in industrialized nations are considered to be shift workers (5). Experiments focused on the effects of shift work indicate that shift workers show an increased prevalence of obesity and that they gain more weight than workers engaged in a conventional workday. In one study, shift workers showed a higher body mass index (BMI) compared with normal shift workers, an increase that did not correlate with age or length of time at the nonconventional shift work (44). Other studies generally support this trend, although in some cases a correlation has been found between length of time at the shift work and BMI (reviewed in Ref. 58). One study tracked male, Japanese workers for up to 27 years to assess whether the night shift population within the manufacturing sector showed an increased risk of obesity relative to their male counterparts who worked only the day shift (128). An increase in obesity was observed in night shift workers, and after 10 years of follow up, a pronounced risk was observed. A similar study designed to assess the effects of alternating shift work on body weight showed that job schedule was associated with BMI but that drinking habits and age were negatively correlated with increases in BMI (229). Cross-sectional data accumulated from the Västerbotten intervention program supports these results. Data collected from shift workers participating in health surveys at ages 30, 40, 50, and 60 years of age indicate that obesity is more prevalent in female shift workers of all ages. Men in some age groups also showed a propensity for obesity (117). Furthermore, high-density lipoproteins were generally decreased in younger shift workers and after adjusting for age and socioeconomic factors, a high level of circulating triglycerides was a risk factor in both sexes. These studies clearly demonstrate a correlation between night shift work and metabolic disturbance, but may even underestimate the damaging effects of circadian dysrhythmia as workers having difficulty adapting to night shifts are often moved back to daytime shifts and are therefore removed from analysis such as these (128).

Unarguably, shift workers have a hard time adjusting completely to a new phase of zeitgebers (198). A new phase of schedule means that the phase of endogenous rhythms will
need time to adjust. For example, food consumption generally takes place during the waking nighttime hours in night shift workers, often resulting in an additional meal consumed during the 24-h day. This requires peripheral clocks to adapt and alter the phase of humoral rhythms such as those of ghrelin. The brain must also adapt to a new sleeping schedule during the day, a task that is arduous as some shift workers report (53). Many shift workers complain of fatigue, jetlag type symptoms, gut disturbances, among other circadian-related maladies, implying that desynchrony might last a very long time, because the internal clocks of the body are not cohesive with the new environment (53). Besides the obvious physical manifestations of fatigue, pain, and discomfort, there appear to be further and yet unexplored ramifications of an internal clock that is out of phase with its environment.

One concern with this internal and external desynchrony is that the levels of coronary artery disease associated with shift workers are rising. One study looking at United States female nurses who worked rotating night shifts (defined as equal to or greater than three nights per month as well as additional day and evening shifts) reveals that women who worked 6 years or more of shift work had an increased risk of coronary heart disease after correcting for smoking among other risk factors (120). A recent summary of 17 prior studies focused on shift work and cardiovascular diseases (reviewed in Ref. 122) reveals that shift workers had a 40% increased risk of cardiovascular disease relative to exclusively daytime workers. It is well known that several heart-related conditions manifest more frequently at particular times of the day. Ventricular tachycardia, cardiac arrest, acute myocardial infarctions, and myocardial ischemia have all been reported to manifest in a circadian fashion (reviewed in Ref. 52).

To better understand the mechanisms behind internal synchronization, experiments with rodents have been designed to mimic human night shift work. One such study used a wheel-running task imposed on nocturnal rats either during their normal sleep phase or during their normal wake phase. Interestingly, plasma glucose rhythmicity was lost entirely in rodents that “worked” during their sleep phase and serum triglyceride (TAG) levels were reversed from those of control animals, with peaks occurring during the sleep phase (201). To address whether internal desynchrony was present, PER1 and PER2 protein expression was analyzed in the SCN in control and working groups where it was found both in phase and of comparable amplitude in the SCN of sleep phase-working animals relative to controls. Corticosterone levels were also invariant, other than an initial rise when animals began their work phase, regardless of zeitgeber time. After 3–4 wk of sleep-phase work, rats subjected to work during the sleep phase gained more weight than their counterparts that worked during the waking phase, supporting human studies showing a similar profile of adiposity and weight gain in shift workers. It is likely that such desynchrony also exists in humans. If pharmacological treatments could be devised to help assist in internal and external synchronization, perhaps some of the negative effects associated with shift work might be avoided.

Perhaps a more common form of circadian desynchrony comes in the form of social jetlag, when individuals are not sleeping within the normal circadian sleep window due to time schedules imposed by work or school, social events, etc. Recent studies addressing this common problem show that even social jetlag is associated with obesity (195). Importantly, sleep timing appears to be as important a regulator of body mass index as sleep duration.

### III. Circadian Rhythms in Metabolic Tissues

#### A. How the Brain Talks to the Periphery and Vice Versa

As the synchronizer of rhythms throughout the body, the efferents of the SCN play a paramount role in the circulation of humoral factors that target peripheral tissues. Many SCN efferents are contained within regions of the hypothalamus itself (Figure 3). The core region of the SCN, which assimilates photic and nonphotic input from the retinohypothalamic tract and the raphe, respectively, appears to project predominantly to the lateral subparaventricular zone (207). Conversely, the shell of the SCN, which receives input from the limbic forebrain and other hypothalamic loci, projects predominantly to the medial subparaventricular zone and the dorsomedial hypothalamus (139). In a circuit that includes the paraventricular nucleus and the intermediolateral nucleus in the thoracic spinal cord, the SCN communicates with the pineal gland, using sympathetic neurons of the superior cervical ganglion, thus regulating the rhythmic secretion of melatonin. Melatonin shows peak expression during the dark phase in both nocturnal and diurnal animals (157) and directly feeds back into the clock by targeting receptors in the SCN. The effects of melatonin on physiology are diverse. For example, melatonin appears to have a strong effect on blood pressure, and its receptors are found throughout the blood vessels of many arterial beds (reviewed in Ref. 193). Oscillating melatonin levels probably contribute to the normal circadian variations in human blood pressure. As ambulatory heart rate is a circadian controlled function, melatonin has been used to reduce blood pressure in patients with hypertension (203). The decrease in human blood pressures in both normo- and hypertensive patients after melatonin administration is consistent with rodent studies where melatonin therapy is an effective treatment for rats with spontaneously high blood pressure (230). Melatonin probably provides this effect, in part, because it relaxes the smooth muscle wall.
The SCN uses the autonomic nervous system, including both the parasympathetic and sympathetic systems to regulate the periphery. Glucocorticoids, which are released in a cyclical fashion from the adrenal cortex, are controlled by the circadian clock. Glucocorticoids oscillate in both an ultradian and circadian fashion, with circadian peaks occurring during the early morning for diurnal animals and early evening for nocturnal animals (reviewed in Ref. 46). Adrenocorticotropic hormone (ACTH), which is released by the corticotrope cells of the pituitary, shows a similar profile and stimulates the release of the steroid hormone corticosterone from the adrenal cortex. Light inhibits the release of corticosterone, and in SCN-lesioned animals, light does not produce the normally observed depression of corticosterone release. Corticosterone production is linked to the environment indirectly and probably depends on SCN relays to the paraventricular nucleus (22). The circadian regulation of corticosterone is important for physiology, as corticosterone serves not only as a precursor for aldosterone, but it also functions in rodents and other mammals as a glucocorticoid, playing central roles in liver metabolic function. Interestingly, in SCN-lesioned animals, where at least some of rhythmic liver gene expression is lost, rhythmicity can be restored by the administration of glucocorticoid receptor activation, suggesting the critical nature of SCN-regulated glucocorticoid release in the regulation of peripheral oscillations (191).

Other humoral factors such as vasopressin and acetylcholine are also used to communicate to the periphery by the clock in the brain. Perhaps one of the best known oscillating humoral factors is arginine vasopressin (AVP). Vasopressin is released into the bloodstream by the pituitary, and it affects the periphery by regulating blood pressure and by decreasing water elimination from the kidney during periods of dehydration. Its circadian release by the pituitary into the cerebrospinal fluid is SCN-dependent (208), but it is also released by the SCN itself where it plays an important role in neuronal synchronization within the structure (8, 111). Acetylcholine is another oscillating neurotransmitter that affects the periphery by inducing skeletal muscle contraction while inhibiting cardiac muscle contraction. In vivo microdialysis techniques demonstrate that there is a circadian release of acetylcholine (113). Acetylcholine serves as the primary neurotransmitter for preganglionic sympathetic neurons, ultimately controlling processes as disparate as heart contraction and gluconeogenesis in the liver. Acetylcholine contributes to timekeeping in the central pacemaker as well and can phase shift SCN rhythms via SCN muscarinic acetylcholine receptors (145). Finally, as demonstrated in viral tracking experiments, the SCN uses the sympathetic nervous system to communicate with a number of peripheral tissues. Peripheral injections of pseudorabies virus (useful for the analysis of identifying trans-synaptic circuits) in brown adipose and white adipose
tissues have revealed that the SCN projects via the sympathetic nervous system to these tissues among many others (reviewed in Ref. 13).

The role of communication provided by the periphery to the brain in controlling energy homeostasis is paramount because the periphery releases a large number of factors such as adipokines and hormones that communicate back to the brain. Such signals provide information to the central nervous system that the peripheral demands have been met or need to be met. Leptin and ghrelin are two such hormones. Ghrelin, which is predominantly secreted by a small fraction of cells in the stomach, signals to the brain that the body needs to be fed. It acts on receptors in the brain in a manner that opposes the actions of leptin and stimulates food consumption by driving feelings of appetite and hunger. Ghrelin levels plummet after a meal, whereas in anticipation of a meal, levels rise again. This oscillatory activity produces changes in ghrelin levels of approximately sixfold in the blood (147). Ghrelin is modified by an eight-carbon fatty acid residue that is central to ghrelin’s effects in the brain, and the unmodified and octanoylated forms of ghrelin circulate in the blood (110). Ghrelin is essential for growth hormone release and is highly conserved across mammals. This is accomplished via hypothalamic arcuate neurons that release growth hormone releasing factor (GHRH), which then activates the release of growth hormone from the pituitary. Ghrelin receptors are also present in the pituitary and can contribute to the release of growth hormone during times of starvation (82). Also released by the periphery, glucose and insulin play seminal roles in the central nervous system to regulate energy intake and metabolism. Recent work on insulin signaling in the brain highlights neurons of the ventromedial hypothalamus as insulin-responsive regulators of diet-induced obesity. While the ablation of the insulin receptor in stereotogenic factor 1 (SF-1) cells of the ventromedial hypothalamus (VMH) has no effect on animals fed a normal diet, when challenged with a high-fat diet, animals devoid of the insulin receptor in this region are partially protected from obesity and show enhanced peripheral glucose metabolism compared with WT control mice. Insulin appears to induce hyperpolarization of VMH SF-1 neurons, which reduces their glutamatergic output to proopiomelanocortin (POMC)-expressing neurons of the arcuate nucleus, thereby inhibiting anorexigenic output. When insulin signaling is impaired in this region, anorexigenic output is enhanced, leading to protection against adiposity.

**B. Circadian Rhythmicity Within Metabolic Tissues**

While once thought to be restricted to the SCN, it is now clear that peripheral tissues host clocks and engage in precise and yet entrainable timekeeping necessary for circadian physiology. As measured in rodents using luciferase activity driven by the Per2 promoter, most tissues show circadian rhythmicity that dampens over time in the absence of the SCN (254). Interestingly, rhythmicity in gene expression within tissues is generally unique, generating phase and period-specific oscillations over the circadian cycle. The brain also shows tissue-specific oscillations in gene expression, which can be independent of SCN rhythmicity (83, 84). While some rhythmic genes are tissue specific, others are shared across many tissues. Some of the clock genes (Per2, for example) show rhythmicity across many different tissues as do Bmal1, Rev-erba, and the Cry genes (249). Some unlikely molecular candidates linking the circadian clock to specific metabolic tissues are being revealed, however, and are illuminating new ways by which circadian physiology may be controlled in tissue-specific ways.

The sirtuin family of proteins is becoming recognized for its peripheral and central role in both circadian rhythmicity and metabolism. The sirtuin family is composed of seven family members, some of which are mitochondrial (SIRT3, SIRT4, and SIRT5) and others that are principally cytoplasmic (SIRT2) nuclear (SIRT1, SIRT6, and SIRT7) or expressed in more than one compartment within the cell (91). Sirt2 (silent information regulator 2, the homolog of the mammalian SIRT1) is an NAD+-dependent histone deacetylase, perhaps best known initially for its role in longevity (135, 144), although it is not clear that it contributes to a longer life span in all species (24). Consequently, the mammalian ortholog of Sir2, SIRT1, has emerged as a central component linking the circadian clock to metabolism. SIRT1 is a class III histone deacetylase and differs from the class I and II deacetylases in that it requires NAD+ as a cofactor for its enzymatic activity. SIRT1 breaks down NAD+ during the process of lysine deacetylation producing O-acetyl-ADP-ribose. During fasting, levels of NAD+ are high, and the activity of SIRT1 is elevated (194). However, when energy is in excess, NAD+ is depleted because the rampant flux through the glycolytic cycle promotes the conversion of NAD+ to NADH. Recent studies demonstrate that SIRT1 directly interacts with circadian clock machinery and that its enzymatic activity contributes to robust oscillations of rhythmic genes in vivo (17, 162). Some studies show that the enzyme is constitutively expressed, while others demonstrate rhythmicity in expression (17, 162). What is clear, however, is that its enzymatic rhythmicity is due in part to an oscillation in the levels of the enzyme’s cofactor NAD+. In search of the source of SIRT1’s oscillating activity, it was discovered that CLOCK:BMAL1 directly regulate the nicotinamide phosphoribosyltransferase (Nampt) gene promoter, the activation of which provides its expression (163, 189). This activation is remarkable in that NAMPT provides the rate-limiting step in the NAD+ salvage pathway. In this way, the classical circadian transcriptional loop is linked to an enzymatic feedback loop in which SIRT1’s own activator is produced in a circadian manner by the circadian clock machinery (FIGURE 4) (59).
While initially identified as a histone deacetylase, SIRT1 also targets non-histone proteins. In the fasted state, SIRT1 activity affects the activity of numerous target proteins including many involved directly or indirectly in metabolic homeostasis. These target proteins include PGC-1, FOXO, IRS1/2, LXR, HNF-4α, FXR, RAR, TORC2, BMAL1, eNOS, LKB1, AMPK, and SREBP (reviewed in Refs. 68, 142). SIRT1-mediated deacetylation of PGC-1 activates the protein, and therefore promotes gluconeogenic gene transcription and the inhibition of glycolytic gene transcription in the liver (194). SIRT3 appears to be the major mitochondrial protein deacetylase where it deacetylates targets such as acetyl-CoA synthetase, lecithin-cholesterol acyltransferase, and 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2) and thereby controls the levels of ketone body production (100, 209, 213). While little is known about the circadian regulation of SIRT3, as a consumer of NAD\(^+\), it is likely that SIRT3 activity, like SIRT1, is activated in a circadian manner. The full extent to which the sirtuin family affects tissue-specific oscillations remains to be seen; however, the ubiquitous expression of the sirtuin proteins across metabolic tissues indicates that their roles in the circadian clock are likely to be important for metabolic homeostasis.

Oscillations within the family of peroxisome proliferator-activated receptor (PPAR) genes have been shown to be prominent in several tissues, including the liver, muscle, brown adipose tissue and white adipose tissue (250). As regulators of lipid storage and lipogenesis, hepatic fatty acid oxidation, and ketogenesis, this family of proteins provides an important link between peripheral rhythmicity in metabolic tissues. A key regulator of gluconeogenesis and glycolysis, the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) provides an additional contribution to clock ticking in metabolically active tissues via its regulatory role in Bmal1 gene transcription. Specifically, PGC-1α acts in concert with RORα to activate Bmal1 gene transcription and, in so doing, links metabolism to the circadian clock (146). In Pgc-1α null mice, circadian clock gene expression is altered, and the circadian oscillation amplitudes of oxygen consumption are considerably dampened while total levels of \(\dot{V}O_2\) are elevated across both day and night (146).

1. Circadian rhythmicity in the brain

A) CIRCADIAN OSCILLATIONS IN NEURONS OF THE CENTRAL PACEMAKER. The mammalian SCN responds to light via neurons that extend from the retina of the eye and through the retinohypothalamic tract. The light responsiveness of these neurons provides synchronization of the organism to the surrounding light-dark cycles. When an organism is put in an environment that deviates from the 24-h cycle, the endogenous circadian clock must reset during a process known as entrainment. The entrainment process takes time (days to weeks) and temporarily renders a desynchronization between the brain and peripheral clocks. For example, the disruption of the endogenous circadian clock during travel across time zones gives one the experience of jetlag, as homeostatic and circadian cues are temporarily out of sync. The endogenous circadian clock must entrain to the new light/dark conditions, a process that relies on distinct signaling transduction pathways that lie upstream of the transcriptional translational loops of the clock machinery already described.

While the clock machinery is required to maintain circadian oscillations in the SCN (i.e., Bmal1 knockout animals and Clock mutant mice have altered circadian rhythms in SCN tissue), much of the complexity that lies upstream of these transcriptional activators has been revealed. The SCN receives nonphotic information from the geniculohypotha-
lamic tract, the dorsal raphe nucleus, and the median raphe nucleus (reviewed in Ref. 45). While some redundancy exists, generally the photic input relayed via the retinohypothalamic tract is independent of rods and cones but relies on photosensitive retinal ganglion cells (18). Photic and nonphotic inputs are integrated in the SCN by various signal transduction pathways. Neurons of the SCN respond to pituitary adenyl cyclase activating peptide (PACAP) during the day, which produces neuronal depolarization (93). At night, neurons of the SCN respond to acetylcholine as well as other cGMP-activating analogs (79). While nonphotic SCN resetting can occur via serotonergic innervation of the SCN (217), light exposure during the night can also reset the SCN clock by triggering the release of glutamate from retinal ganglion cells and a resulting activation of NMDA receptors on SCN neurons. The subsequent depolarization of SCN neurons leads to activation of calcium-sensitive adenyl cyclases in the SCN and the production of cAMP. cAMP activates proteins such as the guanine nucleotide exchange factors (EPAC proteins) as well the mitogen-activated protein kinase (MAPK) signaling cascade, which in neurons couples depolarization to transcription via activation of cAMP response element binding protein (CREB). Indeed, organisms exposed to a light pulse at night show a rapid and robust MAPK phosphorylation in the SCN as well as phosphorylation of CREB and activation of CRE-mediated gene transcription (172, 173). There is direct evidence that cAMP oscillations are required for normal circadian rhythmicity (166). Noncompetitive inhibition of cAMP-producing adenyl cyclase enzymes in the SCN severely prolongs locomotor periods in rodents, an event which is abrogated by mutations in the central clock machinery. Furthermore, adenyl cyclase activators can reset the clock, producing robust changes in the amplitude of gene expression, dependent on the circadian time at which activators are administered.

The neuronal signaling that occurs in response to light would be useless without the ability of SCN neurons to properly synchronize with each other. SCN neurons in culture show circadian rhythmicity in firing, although synapses between the neurons are not necessary or sufficient for this rhythmicity (241). In fact, cultured SCN neurons that oscillate in firing rate can vary drastically in phase from neighboring cells. Phase coherence in vivo is thought to be mediated at least in part by the secretion of vasointestinal polypeptide (VIP), which is released from <25% of SCN neurons (8). VIP appears to be dually important for rhythmicity in some of the SCN pacemaker cells and synchrony in others. Interestingly, one of the receptors for VIP, the G protein-coupled VIPR2 (or VPAC2), not only contributes to maintenance of normal circadian rhythmicity but also to metabolic homeostasis. Loss of this receptor interferes with circadian rhythmicity in rodents (94, 102, 212) and reduces metabolic output during the active cycle (15). Vipr2 knockout animals show a reduction in both oxygen consumption and carbon dioxide output during the nighttime waking hours, and much of their food consumption is advanced into the resting period. Additional studies also support the link between VIP signaling and metabolic homeostasis. For example, VIP knockout mice have elevated plasma glucose, insulin, and leptin levels, probably due to the expression of VIP receptors on taste cells which in the absence of VIP affects the tongue’s role as a sensory gate for energy intake (153).

The SCN modulates the circadian release of multiple neurotransmitters and hormones but responds to many of them in turn. For example, the SCN is required for rhythmic melatonin release from the pineal gland, but the SCN also responds to melatonin, and can be reset by the activation of its own melatonin receptors (178). Similar regulation by and feedback to the SCN is observed with other hormones and neurotransmitters as well. While peripheral tissues respond acutely to glucose demands, the brain anticipates glucose demands. It accomplishes this, in part, by communicating with the periphery to release glucose and insulin in a circadian manner. In humans, insulin release is highest during the early morning hours, when the body anticipates upcoming glucose metabolism (19, 131, 232). There is evidence that the SCN participates in maintaining this balance as lesions of the SCN eliminate plasma glucose and insulin rhythmicity (159, 160). These oscillations appear to be independent of a loss of rhythmicity in food intake (131). Circadian oscillations in glucose and insulin production are also controlled by inhibitory and excitatory inputs into the preautonomic neurons of the paraventricular nucleus (PVN) via the SCN. Stimulation of preautonomic neurons of the PVN via the GABAergic antagonist bicuculline produces hyperglycemia but only during the light period, while exciting neurons of the PVN via the glutamatergic agonist NMDA does not show this time-of-day effect (112). The SCN appears to use rhythmic GABAergic projections to control the activity level of sympathetic preautonomic neurons of the PVN as SCN-lesioned animals show no circadian variation in hyperglycemia after GABAergic antagonism.

b) CIRCADIAN OSCILLATIONS IN FOOD ENTRAINABLE ENSEMBLES OF THE BRAIN. As the hypothalamus plays a central role in feeding, how the circadian clock affects hypothalamic function has been a recent focus of interest. Lesion experiments that predate the 1950s reveal that while disruption of the medial hypothalamus causes hyperphagia and obesity, lesions of the lateral hypothalamus lead to a cessation of food consumption (reviewed in Ref. 47). The arcuate nucleus of the hypothalamus plays a unique role in metabolic homeostasis. Within this structure are two neuronal populations that contribute profoundly to energy homeostasis: one population consists of orexigenic neurons [expressing the neuropeptide Y (NPY) and agouti-related peptide (AgRP)] and another population consists of anorexigenic neurons
[which express proopiomelanocortin-derived peptides including the α-melanocyte stimulating hormone (MSH) and cocaine- and amphetamine-regulated transcript (CART) proteins]. Both of these neuronal populations project to the PVN, where AgRP antagonizes melanocortin receptors and α-MSH functions as an agonist at melanocortin receptors (66, 150). Thus injection of NPY into the paraventricular hypothalamus causes an increase in food intake (220). Interestingly, NPY has been shown to oscillate in a circadian fashion, an oscillation which is controlled in part by both serotonin and GABA and is abolished by feeding restriction (80, 246). Serotonin enhances NPY release while GABA (which also oscillates in a circadian fashion) inhibits NPY release in the SCN. The adenylate cyclase-coupled melanocortin receptors respond to these peptides, and knockouts of these receptors have demonstrated their importance in energy homeostasis. Melanocortin 3 (MCR-3) and melanocortin 4 (MCR-4) receptor knockout animals are both obese, with MCR-3 rodents being obese without hyperphagia. MCR-4 mutations are known to cause obesity in both humans and rodents (67, 105). The melanocortin-secreting cells of the arcuate nucleus are highly responsive to leptin (184). The precise mechanisms underlying leptin’s effects on body weight are still unclear, but leptin receptors in GABA-secreting neurons appear to contribute greatly to body weight regulation. Specifically, the elimination of leptin receptors from all GABA-secreting neurons but not glutamatergic neurons promotes dramatic increases in food intake and body fat mass (238).

Ghrelin produces its orexigenic effects by triggering the activation of the growth hormone secretagogue receptor (GHSR). GHSR was first identified as ghrelin-responsive in the pituitary, where its activation triggers the release of growth hormone (123). Ghrelin’s receptors are also abundant in the hypothalamus, and the GHSR-mediated activation of AMPK in hypothalamic neurons culminates in increased mitochondrial fatty acid oxidation within NPY-expressing cells and GABA-mediated inhibition of NPY/AgRP neighbor POMC neurons (124). As a result, feeding is increased in response to ghrelin release from the stomach. This chain of events bears circadian influence at multiple steps. First of all, ghrelin oscillates in a circadian fashion (141). Second, the increased fatty acid oxidation depends on cofactors that have been shown to oscillate in other tissues, such as NAD+ (163, 189). As the molecular machinery required for the feedback loops depicted in FIGURE 4 are intact in hypothalamic circuits, it is quite possible that a similar circadian profile can be expected for NAD+-regulated processes in melanocortin neurons.

2. The hepatic circadian clock

Due to the relative importance of the liver in glucose and lipid homeostasis, its participation in the circadian clock is of central importance to metabolic physiology. In fact, the liver has been a central target in the study of circadian rhythmicity as it displays robust oscillations in circadian output genes as well as in genes specific to the hepatic system (2). Hepatic rhythmicity depends in part on the rhythmic intake of food which is preserved in constant dark conditions. Interestingly, even under the influence of a functional central clock, the vast majority (over 80%) of hepatic genes are rhythmic in response to food intake (237). Conversely, in mice devoid of circadian rhythmicity, such as is the case in Cry1−/−/Cry2−/− double knockout mice, restricted feeding can restore rhythmicity to many genes in the liver that are otherwise arrhythmic in expression. While SCN lesions generally abolish circadian rhythmicity in the liver, strong transcriptional activators, such as the glucocorticoid receptor, can confer rhythmicity to gene expression made arrhythmic by SCN lesions (191). In spite of the influence of food intake on the hepatic clock, the core clock proteins still hold a prominent role in maintaining the hepatic clock. Clock mutations or deletions such as the ClockΔ19 mutation and the Clock exon 5 deletion severely affect the hepatic circadian system as demonstrated in numerous rodent studies (41, 42, 154). Bmal1 knockout mice are arrhythmic in the brain and the liver as are the ClockΔ19 mice (154, 236). Interestingly, Clock knockout mice show some arrhythmia in the liver while remaining rhythmic in the brain, underscoring the tissue-specific role of CLOCK protein in the liver. While Npas2 protein can compensate for loss of Clock in the brain, it does not compensate for loss of CLOCK function in the liver (42, 43).

These results demonstrate rhythmicity of gene expression in the liver, although not to be ignored is the fact that as much as 20% of liver-soluble proteins are subject to circadian regulation (190). In fact, for almost 50% of rhythmic proteins identified in the liver, no oscillation in the corresponding mRNA levels can be observed, underscoring the importance of posttranslational and translational modifications in maintaining hepatic rhythmicity. The hepatic proteome also shows some dependence on the circadian clock machinery as livers from Clock mutant mice and mPer2−/− mice (11) show dampened oscillatory activity for some proteins (190).

Recent work on the hepatic circadian clock has led to an emerging theme in the circadian field; several circadian clock proteins actually have numerous intracellular roles in addition to contributing to the classical loop of FIGURE 2. As many genes show liver-specific oscillations, it is perhaps not surprising that some members of the clock machinery interact with non-core clock proteins such as tissue-specific nuclear factors (FIGURE 5). Nuclear receptors are central to liver metabolism, regulating genes involved in glucose and lipid metabolism among others (251). Recently, PER2 and CRY1 have been observed to have functions independent of their CLOCK:BMAL1 repression (86, 255). Thus far, PER2 seems particularly promiscuous, binding to PPARγ, PPARα, and REV-ERBα (87, 206), thereby controlling white adipose
and liver tissue metabolic processes. PER2 appears to be unique among the PER proteins in its ability to bind core clock machinery as well as these nuclear receptors. Studies in the last decade have confirmed the circadian rhythmicity of numerous nuclear receptors, the oscillations of which show tissue specificity in some cases (250).

CRY1 also appears to have dual roles in regulating the hepatic clock. In addition to its role as a negative-feedback regulator of CLOCK:BMAL1-dependent gene transcription, recently demonstrated is its ability to suppress hepatic gluconeogenesis. CRY1 protein oscillates, with elevated levels in the liver occurring during the nighttime in nocturnal rodents. CRY1 appears to block cAMP production in the liver, probably by binding to Gsα, thereby preventing coupling of G protein-coupled receptors to adenylyl cyclases (255). As forskolin, a general adenylyl cyclase activator, is able to overcome inhibition by CRY1 of gluconeogenic gene expression [phosphoenolpyruvate kinase (PEPCK) and glucose-6-phosphatase (G-6-Pase), specifically] in the presence of more than one G protein-coupled receptor, it is possible that CRY1 is a general inhibitor of Gsα.

3. Circadian rhythmicity in metabolic peripheral tissues

Most tissues display circadian rhythms. In addition to the hepatic clock, other metabolic tissues show strong oscillations in gene expression. Adipose tissue is among these. Adipocytes host molecular clocks, and their proliferation is under the influence of the transcription factor CCAAT enhancer binding protein beta (C/EBPβ), the expression of which oscillates in adipose tissue and by the circadian protein REV-ERBα, which suppresses anti-adipogenic genes (reviewed in Ref. 20). Adipose tissue is particularly relevant to metabolic homeostasis because leptin and adiponectin, two hormones central to the control of metabolism and cardiovascular disease, are released from adipocytes in a circadian fashion. The adipokine adiponectin is also released from adipocytes, and its plasma levels fall during the nighttime hours (78). This circadian property of adiponectin release has been a topic of interest as adiponectin, also known for its anti-inflammatory and antiatherogenic properties, is tightly linked to metabolism and body weight regulation in humans. For example, decreases in adiponectin have been observed in individuals with disorders associated with insulin resistance including obesity and type 2 diabetes (101, 242). The oscillation of adiponectin may also be important for target tissues such as muscle where adiponectin signaling affects the number of mitochondria. Recent studies looking at adiponectin signaling in muscle show that loss of the adiponectin receptor 1 in muscle results in a decrease in exercise capacity (107). Mediated through AMPK and SIRT1 (two proteins that contribute to circadian rhythmicity), adiponectin appears to modulate muscle insulin sensitivity by modulating both PGC-1α expression (via CaMK and CREB) and its activity (via SIRT1-mediated deacetylation). These rodent studies complement what is seen in humans, namely, a reduction in adiponectin receptor 1 and PGC-1α activity in individuals with type 2 diabetes (158).

Leptin secretion by adipocytes is also a circadian regulated event. Leptin’s contribution to body weight regulation was first observed in the ob/ob and db/db mice, two obese, mutant mice generated at Jackson laboratories (31, 104, 106). Subsequent studies revealed that the obese (ob) gene was a leptin-encoding gene, and db/db mice were found to lack the leptin receptor (28, 256). These leptin-deficient mice are hyperphagic, show mild hyperglycemia and severe obesity, and serve as a model for type 2 diabetes. While altered leptin signaling has accounted for only a small percentage of human obesity, its link to circadian rhythms may contribute to the metabolic physiology of these individuals. In humans, plasma leptin levels oscillate with a maximum level occurring in the late night and early morning hours (inverse to adiponectin). Leptin levels generally correlate with increased adiposity, and leptin levels fall after weight loss in both mice and humans (151). Leptin levels as well as oscillation amplitude are greatly enhanced in obese women compared with normal-weight control women (136).
Aside from the humoral oscillations generated by adipose tissue, recent work shows that PER2 plays a key role in adipocyte gene expression via a direct interaction with PPARγ (87). In adipose tissue, the binding of PER2 to PPARγ recruits it away from target gene promoters. Studies performed on Per2−/− mice (10), which have reduced adiposity, show that in the absence of PER2, genes normally expressed in brown adipose tissue begin to be expressed in white adipose tissue (87), essentially transforming white adipose tissue into a highly oxidative, brown adipose-like tissue, enhancing energy output and thereby producing a lean mouse.

The pancreas hosts an autonomous clock, and recent work on pancreatic islet cells clocks demonstrates their importance in insulin production and blood glucose maintenance (189, 199). Specifically, elimination of Clock or Bmal1 function specifically in islet cells of the pancreas causes reduced glucose tolerance, impaired insulin secretion, and alterations in both the size and proliferation of islet cells (152). Importantly, insulin resistance in circadian mutant mice is dissociable from obesity as restoration of islet cell activity can relieve the symptom of insulin resistance in spite of persisting obesity (152).

As a regulator of fluid volume and mineral and electrolyte composition in the body, the kidney has also been studied as a regulator of fluid volume and mineral and electrolyte of persisting obesity (152). The function can relieve the symptom of insulin resistance in spite of insulin resistance in spite of persisting obesity (152).

Circadian rhythmicity within the central nervous system is highly resilient. Even when core components of the clock machinery are genetically disturbed, rhythms typically persist in LD and even DD conditions. This speaks to the persistence of the clock and also to the dependence organisms have on a system that can synchronize with the environment. While disruption of circadian rhythmicity in DD conditions occurs in genetically modified Bmal1 knockout rodents (23), both rodents and humans that harbor certain deletions in, overexpression of, or mutation in other clock genes have produced some (though less pronounced) alterations in the endogenous time-keeping capacities of the clock. More subtle but still debilitating disturbances include loss of rhythmicity in free-running conditions, impaired response to light shift paradigms (i.e., “jetlag” scenarios), and very advanced or delayed sleep cycles. Some of the transgenic and knockout animals made to address the role of circadian clock genes in vivo as well as their corresponding circadian and metabolic phenotypes (or lack thereof) are depicted in Table 2. The number of genetically modified mice demonstrating both circadian and metabolic abnormalities is growing and includes global and sometimes tissue-specific alternations in Bmal1, Clock, Npas2, and isoforms of the Ck1, Cry, Per, Pgc-1, Rev-erb, and Ror genes (23, 35, 41, 125, 134, 130, 152, 199, 233, 236, 258) (3, 12, 26, 29, 33, 42, 51, 54–56, 65, 85, 86, 92, 138, 140, 143, 146, 148, 175, 186, 188, 211, 219, 235, 248, 257).

The Clock gene was originally identified in a screen designed to look for endogenous mutations that result in a circadian phenotype (236). The Clockmutant animals were found to harbor a deletion in the Clock gene that involves a 51-amino acid deletion in the transactivation domain, a mutation that is now known to be antimorphic to CLOCK function (121). This Clock mutant mouse (or Clockmutant mouse) has been well studied and is of particular interest from a metabolic perspective. Clockmutant mice are obese on a high-fat diet, insulin resistant, and show altered plasma triglyceride levels on the two separate backgrounds studied (130, 174, 233). Interestingly, another metabolic phenotype caused by the Clockmutant mouse has also been observed. While in a C57Bl6 background, the Clockmutant mutation confers obesity on a high-fat diet, Jcl:ICR background mice harboring the Clockmutant mutation show no obesity (174), but show abnormal lipid partitioning. When fed a high-fat diet, the Clock mutation in an ICR background reduces hepatic triglyceride levels. Oscillations in serum FFA levels are ablated by this mutation, and under high-fat feeding conditions, serum FFA levels rise (130). While plasma glucose and triglycerides have been repeatedly observed to oscillate in WT animals, oscillations are disrupted in Clockmutant mice (197, 205, 210). The hypertriglyceridemia in Clockmutant mice may be attributable...
to the inability of the circadian clock to adequately regulate diurnal variations in plasma triglycerides. Specifically, oscillating ApoB lipoprotein-carrying triglycerides depend on the microsomal triglyceride transfer protein (MTP) chaperone to shuttle between membranes. SHP (small heterodimer partner) regulates MTP, and the recent discovery of a functional CLOCK-responsive E box in SHP led to studies which revealed its diurnal variation and contribution to normal circadian fluctuations in plasma triglyceride levels (24, 179). Aberrant expression of other metabolism-regulating genes has also been observed in the Clock/H9004 mice on one or the other background, including hypocretin neuropeptide precursor (Hcrt, which encodes for orexin A and orexin B), ghrelin, cocaine and amphetamine regulated

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tissue-Specific Disruption?</th>
<th>Circadian Phenotype</th>
<th>Metabolic Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmal1 (Arntl)</td>
<td>No</td>
<td>Arrhythmia in DD, altered light response</td>
<td>Reduced activity in LD, reduced lifespan and accelerated aging</td>
</tr>
<tr>
<td>Bmal1 (Arntl)</td>
<td>Yes, pancreas</td>
<td>Impaired GT, reduced islet insulin secretion</td>
<td></td>
</tr>
<tr>
<td>Bmal1 (Arntl)</td>
<td>Yes, liver</td>
<td>Fasting-induced hypoglycemia</td>
<td></td>
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<tr>
<td>ClockΔ19</td>
<td>No</td>
<td>Long period in DD, then loss of rhythmicity</td>
<td>Increased serum TGA, impaired glucose sensitivity, obesity, low liver TG on high fat diet, reduced islet size and proliferation</td>
</tr>
<tr>
<td>Clock &quot;deficient&quot;</td>
<td>No</td>
<td>Slightly shorter period in DD, impaired light response and entrainment in LD</td>
<td>Partial diabetes insipidus, decrease in blood pressure, slight decrease in lifespan</td>
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<tr>
<td>Clock/Npas2</td>
<td>No</td>
<td>Arrhythmia in DD</td>
<td></td>
</tr>
<tr>
<td>Ck1c (tau mutation)</td>
<td>No</td>
<td>Shortened period in DD, impaired entrainment</td>
<td></td>
</tr>
<tr>
<td>Ck1δ mutant</td>
<td>No</td>
<td>Shortened period</td>
<td></td>
</tr>
<tr>
<td>Ck1δ</td>
<td>No</td>
<td>Embryonic lethality</td>
<td></td>
</tr>
<tr>
<td>Ck1δ</td>
<td>Yes, liver</td>
<td>Local period lengthening</td>
<td></td>
</tr>
<tr>
<td>Cry1, (Tg)</td>
<td>No</td>
<td>Long period in DD, splitting, impaired entrainment</td>
<td>Enhanced glucose in serum and urine</td>
</tr>
<tr>
<td>Cry1/Cry2</td>
<td>No</td>
<td>Arrhythmia in DD, altered light response</td>
<td>Salt-sensitive hypertension</td>
</tr>
<tr>
<td>Noc (Nocturnin)</td>
<td>No</td>
<td>Enhanced adaptation to light entrainment</td>
<td>Higher nocturnal wheel running, delayed FAA activity</td>
</tr>
<tr>
<td>Per1</td>
<td>No</td>
<td>Shortened period in DD, impaired light response</td>
<td>Impaired lipid metabolism</td>
</tr>
<tr>
<td>Per2</td>
<td>No</td>
<td>Shortened period followed by arrhythmia in DD, impaired light response</td>
<td></td>
</tr>
<tr>
<td>Per3</td>
<td>No</td>
<td>Mild period shortening in DD</td>
<td>Increased adipose mass, glucose intolerance</td>
</tr>
<tr>
<td>Per1/Per2</td>
<td>No</td>
<td>Arrhythmia in DD</td>
<td></td>
</tr>
<tr>
<td>Pgc-1α</td>
<td>No</td>
<td>Mildly longer period in DD</td>
<td>Resistance to HF diet-induced obesity, reduced thermogenesis</td>
</tr>
<tr>
<td>Pgc-1β</td>
<td>No</td>
<td>Reduced activity in the dark</td>
<td>Reduced serum TG and FFA levels, elevated hepatic lipid accumulation during HF feeding</td>
</tr>
<tr>
<td>Rev-erbα</td>
<td>No</td>
<td>Mildly shortened activity period in DD, impaired light response</td>
<td>High plasma LDL, altered hepatic TGA levels, low bile acid accumulation</td>
</tr>
<tr>
<td>Rev-erbα, Rev-erbβ (inducible)</td>
<td>No</td>
<td>Advanced phase angle of entrainment, short period in DD</td>
<td>Increased circulating glucose and TGA levels, reduced circulating FFA</td>
</tr>
<tr>
<td>Rora (staggerer sg/sg)</td>
<td>No</td>
<td>Mildly shortened period in DD</td>
<td>Increased HDL and hepatic TGA, accelerated development of atherosclerosis, resistance to HF diet-induced obesity</td>
</tr>
<tr>
<td>Rorβ</td>
<td>No</td>
<td>Long period in DD</td>
<td></td>
</tr>
</tbody>
</table>

The circadian mutation as well as the affected tissue is documented along with the resulting circadian phenotype. If metabolic phenotypes for the corresponding mutant animal have been observed, they are listed in the last column.
The ability to tell time revealed that the CLOCK protein (43). Studies addressed to determine why demonstrate a dependence of peripheral oscillators on tissues of the central nervous system (42). The NPAS2 protein was essential for maintaining rhythmicity in would be expected if the presence of either CLOCK or Npas2/Clock double knockout animal, which has no functional CLOCK or NPAS2 protein show arrhythmia, as Npas2/Clock paralogs (the Clock paralog which is highly expressed in the brain and able to bind to CLOCK's transcriptional partner, BMAL1) is able to compensate for CLOCK in the brain but not the periphery. The Clock/Npas2 double knockout animal, which has no functional CLOCK or NPAS2 protein show arrhythmia, as would be expected if the presence of either CLOCK or NPAS2 protein was essential for maintaining rhythmicity in tissues of the central nervous system (42). The Clock knockout mouse is not completely immune to disturbances in the brain's clock, however. These animals show an impaired response to light shifting. Specifically, while light exposure during the early night (ZT12–16) phase delays the circadian clock in WT animals, Clock knockout animals are not phase delayed by light exposure (41). CLOCK and NPAS2 both appear to contribute to sleep homeostasis. Clock mutant mice sleep less and show reduced rapid eye movement (REM) sleep recovery after sleep deprivation than their WT counterparts (165). NPAS2 also has an effect on neuronal energetics as the loss of NPAS2 produces alterations in sleep homeostasis, altering the electrophysiological properties of neurons after sleep deprivation (55, 74).

The phenotype of Bmal1 knockout mouse is unique in that it is the only circadian mutant that harboring a single gene mutation, shows complete arrhythmia (23, 125). Bmal1 knockout mice are completely arrhythmic in DD and display a severe metabolic phenotype. The fat, muscle, bone, spleen, kidney, testis, heart, and lung of Bmal1 knockout animals all show an age-dependent reduction in size, which is consistent with their elevated ROS levels (125). Unlike WT animals, glucose and triglyceride levels do not oscillate in Bmal1 knockout animals, and while WT animals typically show a circadian rhythmicity in blood glucose recovery following insulin injection, such rhythmicity is absent in Bmal1 knockout mice, which show instead pronounced hypoglycemia around the clock in response to insulin (197). These knockouts also show impaired gluconeogenesis, probably due in part to the lack of oscillatory PEPCK activity in their hepatocytes.

The Cry1/Cry2 double knockout animals demonstrate the necessity for cryptochrome protein function in the circadian clock in the absence of zeitgebers. These animals show complete arrhythmia in free running conditions (235). These mutants also show disrupted rhythms after changes in the lighting conditions. This particular attribute of the Cry1/Cry2 double knockout animals was somewhat of a surprise at the time the experiments were done as cryptochromes were still thought to be the primary light sensors for the mammalian biological clock. [Since this time, the photopigment melanopsin has been demonstrated to be important for photosensitive retinal ganglion cells in relaying light-sensitive information to the brain (196).] In addition to the Cry1 and Cry2 knockout animals, Cry1 overexpression in vivo has also been used to address the role of Cry1 in circadian timekeeping. Two strains of overexpressing Cry1 mice have been made: Cry1 (Tg) animals, which overexpress WT mouse Cry1; and Cry1 (AP-Tg) animals, which overexpress a Cry1 mutant lacking a conserved cysteine proline motif (175). While mice overexpressing WT Cry1 show a normal circadian profile, Cry1 (AP-Tg) mice show long periods in free running conditions. Furthermore, Cry1 (AP-Tg) mice show elevated levels of serum and urine glucose. In addition to the high glucose levels, these mice show some common symptoms of diabetes mellitus, polydipsia and polyuria.

Cry1/Cry2 null mice show an additional metabolic phenotype not yet observed in other circadian mutants, that of salt-sensitive hypertension. Hypertension is a common malady in humans that substantially increases the risk of stroke and heart attack. There are a number of known risk factors for salt-sensitive hypertension including age, race, family history, tobacco use, stress, and physical inactivity. Relatively recent data reveal that an abnormal circadian rhythm may be added to the list. Specifically, Cry1/Cry2 mutant mice show a malfunctioning renin-angiotensin-aldosterone system (RAAS) resulting from the aberrant production of the mineralocorticoid aldosterone in the zona glomerulosa cells of the adrenal gland. The 3β-hydroxysteroid dehydrogenase-isomerase enzyme oxidoreductase family is required for aldosterone synthesis and is dysregulated in the adrenal gland of Cry1/Cry2 mutant mice. Expression of the Hsd3b6 isoform not only oscillates in WT animals but is elevated and void of oscillatory expression in the zona glomerulosa of Cry1/Cry2 mutant mice. It is still unclear why Cry1/Cry2 mutant mice show this phenotype. However, the derepression of CLOCK:BMAL1-mediated gene transcription in the absence of Cry1 may allow for enhanced clock output expression. There are binding sites for the CLOCK:BMAL1-responsive DBP gene in the promoter of Hsd3b6 which may facilitate its increased expression (FIGURE 6).
While metabolic phenotypes are observed in the Bmal1 knockout mice as well as ClockΔ19 animals, tissue-specific clock targeting has been insightful in understanding the ways in which circadian processes affect metabolism. Mice generated to lack Bmal1 in pancreatic islet cells only demonstrate the importance of the clock in insulin secretion. Islet cell-specific Bmal1 knockout mice have normal body weights and compositions but show elevated glucose levels in ad libitum conditions. In addition, these mutants have impaired glucose tolerance and reduced insulin secretion. Islet studies from these mice confirm the reduced insulin responsiveness to glucose, directly implicating the pancreas clock as a contributor to normal insulin sensitivity and protection against diabetes mellitus (152).

In addition to its role in insulin secretion, BMAL1 plays a unique role in adipose tissue development. BMAL1 contributes to the process of lipogenesis and adipocyte differentiation (214). Mouse embryonic fibroblasts lacking Bmal1 expression are unable to differentiate into adipocytes, a process that is at least partly recoverable by the adenoviral expression of Bmal1 (full recovery requires the exogenous addition of PPARγ ligand). Furthermore, metabolic labeling experiments of Bmal1-overexpressing adipocytes have demonstrated that the lipid synthesis activity in modified adipocytes is much higher, allowing the cells to accumulate unusually large lipid deposits (214).

Rodent knockouts of nocturnin, a circadian deadenylase that is expressed with high amplitude in the liver, show negligible circadian defects but rather exhibit remarkable changes in metabolism. Noc−/− mice are resistant to diet-induced obesity, possibly due to decreased food absorption from the intestine (85). Lipogenic gene expression is reduced in Noc−/− mice, correlating with a decrease in hepatocyte lipid accumulation.

IV. MOLECULAR RHYTHMICITY OF METABOLITES AND THEIR REGULATORY ENZYMES

The identification of molecular rhythms that link the clock to metabolism has rapidly expanded from what was known about those present in the nucleus (via the clock transcriptional machinery) to those occurring outside of the nucleus (167, 168). While many genes oscillate in expression, post-translational modification of proteins provides an additional oscillatory, nonnuclear event that can create rhythmicity in enzymatic activity whether or not a corresponding gene transcript oscillates (162, 166, 168, 189, 190). While such events are unlikely to be completely independent of circadian nuclear transcriptional events, the evidence of rhythmicity beyond the level of transcription expands the arena for potential zeitgebers that assist in entrainment or maintenance of local circadian clocks.

A. Rhythmicity in Metabolic Enzymes

Rhythmicity within metabolic pathways relies on oscillations of the enzymes (or their activity) required for a particular metabolic pathway, fluctuations in metabolite production and concentration, or both. It is likely that both oscillations in gene expression, and protein stability as well as metabolite production or turnover work together to maintain rhythmicity within

![FIGURE 6](http://physrev.physiology.org/)

**FIGURE 6.** Possible molecular mechanisms observed for the salt-sensitive hypertension observed in Cry1/Cry2 double-knockout animals. Elevated DBP-mediated gene expression may be responsible in the Cry1/Cry2 double-knockout animals for the elevated Hsd3b6 observed in these animals.
a pathway. An example of such a scenario can be observed in the case of oxidative stress and the mechanisms by which the body copes with increased levels of it. The oxidative stress theory of aging purports that the accumulation of ROS-induced damage within biologically important molecules during an organism’s life leads to aging. Interestingly, many antioxidant enzymes oscillate in a circadian fashion and with a profile that meets the metabolic demands of a particular tissue. For example, during the dark phase, oxidative metabolism is at its highest in the brain of rodents. This corresponds with increased levels of lipid peroxides in the hippocampus. The highest levels of hippocampal catalase and glutathione peroxidase activity, however, are concomitant with nocturnal peaks in liperoxidation, a coordination that probably functions to protect this memory-encoding structure from extensive oxidative damage over time (72). In this situation, the mRNA, protein, and activity levels of catalase and glutathione peroxidase oscillate in a circadian fashion. Other enzymes, whose activity and/or expression oscillates in a profile that is consistent with circadian physiology, include those important for cholesterol metabolism, amino acid regulation, glucose metabolism, drug and toxin metabolism, and the citric acid cycle (reviewed in Ref. 39). These and other evidence supporting a circadian link to metabolism and aging will be discussed further.

1. Rhythms in enzyme expression

The expression of many metabolic enzymes appears to be under circadian control. Sometimes rhythmicity in enzyme abundance is due to oscillatory expression of its mRNA, but sometimes the mRNA doesn’t oscillate while the protein does (190). This is likely due to the rhythmic stabilization or degradation of specific proteins. Regardless of the mechanism, the implications of rhythmic enzyme production are numerous. For example, the mRNA abundance of several mitochondrial complex I proteins oscillate (reviewed in Ref. 137). Interestingly, the expression within the SCN of ~20 protein subunits involved in the respiratory chain reaches its peak just before dawn, this occurring just prior to the circadian peaks for neuronal firing in the region, when the metabolic state within SCN neurons would be at its highest (9, 180). Gene expression arrays have revealed robust circadian oscillations in genes involved in detoxification, stress response, and other metabolic proteins. Interestingly, the activity of some of these gene products is driven by NAD⁺ or NADP⁺. Examples include short-branched chain acyl CoA dehydrogenase, aldehyde dehydrogenase, and oxido reductase (25). Enzymes necessary for detoxification comprise a large number of cycling genes in the fly head, including six cytochrome P-450 proteins as well as glutathione-S-transferase. The fly body also hosts circadian oscillations in gene expression, including the oscillations of a number of redox-associated genes. Consistent with the Drosophila microarray data, in the rodent brain and liver, glutathione peroxidase production peaks in a circadian manner. This event is driven by melatonin and is quite possibly temporally organized to specifically scavenge for reactive oxygen species (ROS) as ROS abundance peaks just before glutathione per-oxidase production reaches its highest (137). Other metabolism-associated genes also show circadian oscillations in expression. These include genes encoding the glucagon receptor, glucokinase, glucagon, Glut2, glucose-6-phosphate transport protein, pyruvate kinase, and pyruvate dehydrogenase (131, 132, 180). Other metabolic enzymes such as glucose-6-phosphatase, acetyl-CoA carboxylase, cytochrome oxidase, lactate dehydrogenase, fatty acid synthase, and glycogen phosphorylase have also been shown to be rhythmically expressed and/or activated (reviewed in Ref. 76). These data support the idea that the cellular metabolism is greatly influenced by the rhythmic production and activity of mitochondrial proteins. This cross-talk likely determines how the cell responds in a temporally restricted manner to DNA damage induced by accumulating oxidative stress.

Two enzymes that assist in translating the metabolic cues in the cell to the clock were recently shown to oscillate in expression. First, the enzyme that controls the rate-limiting step in the NAD⁺ salvage pathway, NAMPT, was shown to oscillate in a CLOCK:BMAL1- and SIRT1-dependent manner (163). This oscillation is potentially of significance for all NAD⁺-dependent biochemical processes as it could be one mechanism by which the circadian clock temporally controls all NAD⁺-dependent reactions. Second, the AMP-activated protein kinase (Ampk), an important mediator of metabolic signals, was shown to oscillate. Specifically, oscillations in one of its regulatory subunits, ampkβ2, occur in a circadian fashion and are likely responsible for the rhythmic nuclear translocation of AMPK where it has access to specific substrates including CRY1 (133). The oscillation of AMPK is important in linking circadian rhythms to metabolism as AMPK activity is a direct reflection of the cell’s energy status.

The expression of serotonin N-acetyltransferase (Aanat) in the pineal is important for the rhythmic release of melatonin as it serves as the rate-limiting step in melatonin synthesis. The robust oscillation of this enzyme depends in large part on the yin-yang effect of CREB and CREM-mediated promoter activity (reviewed in Ref. 73). While cAMP activates CREB-mediated gene transcription at the Aanat promoter, cAMP can also inhibit expression of the enzyme through the protein ICER. ICER is generated by the use of an alternative promoter in the Crem gene, and it oscillates robustly in the pineal where it helps control melatonin output by repressing Aanat gene transcription (221). Interestingly, ICER can regulate its own transcription as it binds to a CREB response elements in its promoter with high affinity. The control of melatonin by AANAT represents how oscillators on different levels coordinate control physiology (FIGURE 7). Melatonin is released in a circadian fashion by the pineal to control physiological events as disparate as heart rate and sleep homeostasis. This requires synchronization between individual oscillators of the pineal. Within each of these pineal clocks are oscillations in the transcription of genes involved in melatonin synthesis (i.e., Aanat). However, at the center of Aanat expression lie tran-
scriptional regulators that generate oscillatory rhythms of activation or repression by regulating their own promoters (i.e., ICER). Thus melatonin-generated rhythms are supported by several levels of oscillating events that coordinate remarkably to control this circadian phenomenon.

The plasminogen activator inhibitor type 1 (Pai-1), a key regulator of fibrinolysis, shows an oscillatory expression in plasma and tissues, and the peak hour coincides with the highest incidence of myocardial infarction and high blood pressure. Pai-1 is regulated in part by REV-ERBα-mediated repression and therefore is directly subject to regulation by the clock machinery (240). High levels of Pai-1 are directly related to increased thrombosis, and elevated levels of Pai-1 have been observed in the atheroma taken from patients with type 2 diabetes (218).

2. Rhythms in enzymatic activity

Not all rhythmicity in enzyme activity is driven by oscillations in gene expression for that enzyme. On the contrary, some enzymes show oscillatory activity in the absence of oscillatory expression. For example, activity of the NAD⁺-dependent poly(ADP-ribose) polymerase 1, (PARP-1, an ADP ribosyltransferase) oscillates in a circadian manner, while its abundance appears relatively constant over the 24-h cycle (7). While known to participate in the plant circadian clock (49), recent evidence that its activity is im-
important for the mammalian clock underscores the importance of fluctuations in NAD$^+$/NADH for mammalian circadian physiology. Not only does auto-ADP-ribosylation of PARP-1 occur in a circadian fashion, peaking in the early rest period of nocturnal rodent liver, but PARP-1 directly binds to and poly(ADP-ribosyl)ates CLOCK in a circadian fashion, thereby modulating CLOCK:BMAL1 DNA binding affinity. Interestingly, Parp-1 knockout rodents are impaired in their ability to entrain to food, and while FAA activity appears to be normal, locomotion during the animals’ active period is significantly increased in PARP-1 knockout mice (7).

Posttranslational modifications of enzymes are often important regulators of their activity. Protein acetylation is one such modification that has been observed to modulate protein activity. Numerous acetylated proteins have been assessed by proteomic studies, and such studies show that many of these acetylated sites are highly conserved between rodents and humans. Across different tissues, however, the location of these acetylation events can be quite disparate. Interestingly, numerous proteins involved in metabolic enzymes are acetylated. For example, PEPCK is subject to both transcriptional regulation and the posttranslational modification of acetylation. Acetylation of PEPCK appears to be related to the blood glucose levels and functions to control the rate of PEPCK turnover in the cell by triggering ubiquitination and degradation of the protein (109). While its acetylation has not been looked at in the context of circadian rhythmicity, its acetylation is likely circadian as glucose levels undergo circadian rhythmicity. PEPCK is acetylated by p300, an event that is counteracted by the sirtuin protein family member SIRT2 (109). Interestingly, many metabolic enzymes are acetylated based on fluctuations in intracellular glucose or acetate levels. As acetylation is directly linked to the circadian clock by the sirtuins among other HDACs, it is surmisable that posttranslational modification of these proteins occurs in a circadian fashion to accomplish the roles required of them throughout the circadian cycle. CLOCK may contribute to the circadian cycle of acetylation and deacetylation as its role as a HAT implicates it in direct regulation of its protein targets (50).

AMPK is an important mediator of metabolic signals, and its activity appears to be circadian (40). Recently, using mass spectrometry and bioinformatics, CRY1 protein was found to be directly phosphorylated by AMPK. This phosphorylation event by AMPK is critical for CRY1 turnover in vitro and in vivo and is therefore necessary for normal circadian rhythmicity. This link provides yet another example of the connection between the metabolic state of the cell and circadian gene transcription, in which AMPK acts as a chemical sensor for the cell (133).

B. Rhythmic Metabolite Production and Use

While a few individual metabolites have been shown to oscillate in vivo and in vitro, studies are beginning to focus on more global oscillations within the metabolome. This approach is important because if food can entrain peripheral clocks, it is likely that numerous metabolites that occur as a result of or in preparation for food digestion act as potent zeitgebers in and of themselves. Studies addressing the role of the circadian clock in controlling metabolite levels demonstrate that much of the liver metabolome is under circadian control, for example. Many metabolites oscillate in a circadian fashion, and the oscillation of some metabolites appears to be largely dependent on the expression of Clock (60). Large-scale mining of the literature has been instrumental in mapping these circadian metabolites amid the backdrop of their intracellular surroundings. For example, maps have now been created for circadian (and non-circadian)-controlled metabolites, which incorporate data from multiple sources such as those containing data on circadian gene oscillation of metabolite-producing and/or -degrading enzymes (103), high-affinity transcription factor binding sites for metabolic enzymes (34, 245), and the metabolite’s own interacting metabolites (60, 114–116). Such networks can be visualized on an interactive database, http://circadiomics.igh.uci.edu/, which is freely available.

As large-scale metabolomics becomes increasingly used, the creation of metabolite datasets will be informative with regard to how small metabolites reciprocally interact with the molecular components of the circadian clock. Metabolites representing numerous metabolic pathways appear to be under circadian control, with amino acid and xenobiotic metabolites tending to peak at night in nocturnal rodents and carbohydrate, lipid, and nucleotide metabolites peaking during the rest period (60) While over half of the metabolome may be under circadian influence, other studies in humans which have eliminated circadian activities (such as sleeping, diurnal eating patterns, and activity) from test subjects reveal that a remarkable 15% of the metabolome still remains under circadian control (36). The oscillations of numerous amino acids and urea cycle metabolites appear to be rigidly tied to the circadian clock and have now been reported in several studies (36, 60, 108, 155).

Additional studies focused on the oscillatory nature of specific metabolites have been revealing in how small biochemicals might function as zeitgebers for cells. For example, oscillation of NAD$^+$ has pleiotropic effects in the cell and feeds back into the clock system via CLOCK:BMAL1-mediated gene transcription (163, 189). Within the mitochondria, ATP and NAD$^+$ are used as carrier molecules for oxidation-reduction reactions. These carrier molecules are essential for cellular energy balance. For example, the release of electrons and a hydrogen atom off of substrate molecules are picked up by NAD$^+$ and ultimately used by the cell to generate additional ATP and to drive ATP-dependent reactions. NAD$^+$, which is generated from niacin, par-
addition to its role in ATP generation, NAD+ also provides the ADP-ribose substrate necessary for the ADP ribosylation of target proteins. Prolonged increases in the activity of the ADP-ribose (cADPR) production.

The small molecule cADPR, which is produced from NAD+ by ADP-ribosyl cyclases, has been reported to oscillate in a circadian fashion. cADPR serves as a ligand for the type 3 cyclic ADP-ribose (cADPR) production.

A recently discovered function of the CRY protein appears to also depend on rhythmic metabolic factors. In Drosophila, CRY controls neuronal firing rate. ILNv neurons, which are light-detecting neurons of Drosophila pacemaker cells, depend on CRY for a rapid response to light (69). This is achieved through a redox-based flavin mechanism, which appears to induce CRY-dependent neuronal responses to blue light. This TIM-independent CRY response can be attenuated by the administration of an antagonist, which blocks the flavin binding site in CRY. Potassium channel conductance appears to molecularly couple flavin-bound CRY to alterations in membrane potential as inhibitor cocktail of both voltage-gated and inward-rectifying potassium channels blocks changes in membrane potential in response to blue light while saturating doses of tetrodotoxin do not. Whether flavin oscillates in the Drosophila pacemaker cells is not currently known; however, the activity of CRY in response to light oscillates in a metabolite-dependent way and suggests the oscillatory pattern of FAD itself. The oscillation of FAD in rodent liver suggests that its oscillation may be present in other tissues and organisms (60).

Another route of coupling between metabolite availability and the circadian clock was shown several years ago and involves the ability of NPAS2 to function as a gas sensor (48). Specifically, the PAS domain of the NPAS2 protein binds heme, enabling it to function as a carbon dioxide-sensitive transcription factor. That two enzymes involved in heme and CO biosynthesis are differentially expressed in regions of the brain highly expressing NPAS2 may be more than mere coincidence. The expression of aminolevulinic acid synthases (Alas), a rate-limiting enzyme in heme biosynthesis, oscillates in a circadian fashion. In addition, heme oxygenase 2 (HO-2) involved in CO generation is highly expressed in the forebrain, where high levels of NPAS2 are also observed. If NPAS2 is CO responsive, it may be that both the availability of CO as well as the oscillating mechanism for its production may directly affect the core clock output, namely, NPAS2:BMAL1 dimerization and activation of gene transcription. Interestingly, NPAS2 functions as more than just a CO sensor. Both heme-bound and heme-less NPAS2 PAS domains require a high NADPH/NADP ratio for sufficient DNA binding. Heme is also a ligand for REV-ERB/α (187, 252), the interaction of which precipitates the recruitment of the NCoR/histone deacetylase 3 (HDAC3) corepressor complex that leads to the repression of PGC-1α expression. Itself a potent inducer of heme synthesis, PGC-1α provides a negative feedback loop that maintains heme levels and regulates cellular energy metabolism (243). The contribution of NCoR to the circadian clock has been well demonstrated. NCoR and the HDAC3 complex are recruited to the Bmal1 promoter by REV-ERBα, a process that contributes to the intracellular levels of BMAL1 in the cell. The formation of this complex is necessary for normal circadian rhythmicity as well as the oscillation of numerous metabolic regulatory genes (4, 253).

As NAD+ signals to the cell that the energy state is low, so does AMP. When energy is burned, AMP is produced. Like NAD+, AMP concentration directly links energy to the circadian clock. When AMP levels are elevated, AMPK gets phosphorylated by liver kinase B1 (LKB1) (96). As previously discussed, AMPK was recently identified as playing a direct role in circadian regulation in the form of regulating the stability of CRY1 protein (133). Indeed, mice injected with AMPK activators show decreased levels of CRY1 in the liver, and the absence of LKB1 in the liver of mice allows CRY1 stabilization. AMPK phosphorylates CRY1, which causes CRY1 to bind to the protein F-box and leucine-rich repeat protein 3 (Fbxl3). This F-box protein, which is part of the ubiquitin protein complex responsible for the phosphorylation-dependent degradation of proteins by ubiquitination, degrades both CRY1 and CRY2 (215). CRY1 degradation has the net effect of derepressing CLOCK target genes; however, AMP levels affect gene expression in a more global fashion, via its regulation of CRY1.

While the expression of numerous nuclear hormone receptors is known to oscillate, many do not oscillate. Regardless
of receptor oscillation, a number of endogenous ligands for these receptors do oscillate in a circadian fashion and therefore contribute to the growing number of metabolites and biochemicals that may function as zeitgebers for the clock in different cells and tissues (216, 250, 251). In addition to heme, other nuclear receptor ligands appear to be under circadian regulation. Fatty acids, bile acids, prostaglandins, leukotrienes, vitamins, and hormones appear to be part of the dynamic circadian metabolome that contributes to circadian physiology (60). How these biochemicals function to generate circadian function is distinct and probably tissue specific. A better understanding of how the circadian metabolome changes in different nutrient conditions promises to be important for future understanding of how the energy state of the cell couples to the circadian clock.

V. FROM METABOLITES TO PHYSIOLOGY

There are several physiological circuits that draw attention to the reciprocal interaction of the circadian and metabolic programs in vivo. These include circuits between multiple tissues such as occur between the SCN and the pineal, or the SCN and adipose tissue. They also include feedback loops that rely on humoral signals for interplay such as occur between the gut and the hypothalamus, or the liver and the SCN. For example, the hypothalamus responds quickly to humoral signals released in a circadian fashion by the periphery (such as leptin or ghrelin) and, in turn, regulates the energy output of peripheral tissues. Within these larger systems circuits, however, smaller circuits exist, such as the internal feedback loops within tissues. This lower tier of circuitry includes positive and negative feedback between SCN network neurons (via VIP, for example) or reciprocal regulation of neurons of the arcuate nucleus (such as occurs between NPY/AgRP GABA-releasing neurons and their POMC counterparts). Figure 7 depicts a few of the circuits that operate within the context of broader circuits that are important for the maintenance of energy balance. Finally, subcellular level oscillations include the cellular feedback mechanisms necessary for maintenance of circadian physiology. These include the molecular players involved in the basic feedback loop of Figure 2, but also supporting oscillatory intracellular activity such as occurs during the CREB and CREM-mediated regulation of Aanat expression (see sect. IVA1). If these basic cellular oscillators are faulty, circadian physiology is compromised because communication on the systems level is supported by the rhythmic activity of single cell oscillators.

As the rise in metabolic disorders is occurring at an unprecedented rate, understanding how the circadian system affects metabolic homeostasis has been a focus of numerous research communities in the last century. Lifestyles that affect metabolic homeostasis has been a focus of numerous research communities in the last century. Lifestyles that affect metabolic homeostasis has been a focus of numerous research communities in the last century. Lifestyles that affect metabolic homeostasis has been a focus of numerous research communities in the last century. Lifestyles that affect metabolic homeostasis has been a focus of numerous research communities in the last century. Lifestyles that affect metabolic homeostasis has been a focus of numerous research communities in the last century. Lifestyles that affect metabolic homeostasis has been a focus of numerous research communities in the last century. Lifestyles that affect metabolic homeostasis has been a focus of numerous research communities in the last century. Lifestyles that affect metabolic homeostasis has been a focus of numerous research communities in the last century. Lifestyles that affect metabolic homeostasis has been a focus of numerous research communities in the last century. Lifestyles that affect metabolic homeostasis has been a focus of numerous research communities in the last century. Lifestyles that affect metabolic homeostasis has been a focus of numerous research communities in the last century. Lifestyles that affect metabolic homeostasis has been a focus of numerous research communities in the last century. Lifestyles that affect metabolic homeostasis has been a focus of numerous research communities in the last century.

that metabolism and circadian rhythms are tightly linked. The goal, therefore, is to gain further insights into how these processes converge and to provide useful environmental, behavioral, or pharmacological solutions that curb the rising rate of metabolic disorders associated with circadian disturbance.

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DISCLOSURES

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METABOLISM AND THE CIRCADIAN CLOCK CONVERGE


