Chapter 13
Sleep, Circadian Rhythms and Metabolism

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Introduction

Obesity and cardiometabolic disease are closely linked disorders that have recently accelerated throughout the industrialized world, coincident with more sedentary lifestyle and poor nutrition; however a complete understanding of the environmental precipitants underlying metabolic disease remains obscure. Mounting evidence from epidemiological studies has pointed towards a novel yet less appreciated factor that correlates with the recent expansion of these epidemics, namely, the introduction of artificial light and work at night-time, in addition to the rise in sleep curtailment. At the physiological level, it has been well-documented that many processes, including glucose and lipid metabolism, body temperature, and corticosterone production vary in a circadian fashion; moreover, there is an established temporal variation to health catastrophes such as myocardial infarction, cerebrovascular accident, and hypertensive crises. Over the past decade, major advances have emerged in our understanding of the underlying molecular mechanisms linking circadian rhythms, sleep, and metabolism, primarily through studies in experimental genetic models that became available following the landmark discovery of the first mammalian circadian clock gene *Clock* in 1997 [1, 2].

In this chapter, we highlight evidence at the intersection of clinical medicine and experimental genetics that illustrates how perturbations of the internal circadian system, and alterations in clock gene function, participate in the onset and progression of obesity and related disorders. An exciting aspect of the field has been the integration

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of behavioral and physiological approaches and the emerging insight into integration of neural and peripheral tissues in disease pathogenesis.

Molecular Relationships Between Metabolism and Circadian Rhythms

The Core Clock Molecular Network: The Basis of Circadian Rhythms

Circadian rhythms regulate a wide variety of physiological and metabolic functions in most organisms [3, 4]. At the molecular level, a network of autoregulatory coordinated transcription–translation feedback loops regulates the core molecular clock, maintaining approximately 24 h rhythmicity in order to match the Earth's rotation around its axis.

In mammals, the positive elements of these loops include members of basic helix-loop-helix (*bHLH*)-PAS (*Period-Arnt-Single-minded*) transcription-factor family of the transcription factors CLOCK (*Circadian locomotor output cycles kaput*), its paralogue NPAS2 (*Neuronal PAS domain protein 2*), and BMAL1/ARNTL (*Aryl-hydrocarbon receptor nuclear translocator-like*). CLOCK or NPAS2 and BMAL1 heterodimerize to activate the rhythmic transcription of genes containing E-box enhancer sequences, including the *Period* (*Per1*, *Per2*, and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) genes (Fig. 1). The PER and CRY proteins comprise the negative limb of the feedback loop; upon translation, PER and CRY proteins multimerize and subsequently translocate to the nucleus and directly inhibit the transcriptional activity of the CLOCK:BMAL1 complex (Fig. 1). Posttranslational modifications, including phosphorylation and ubiquitination, provide further regulation of the clock network. *Casein kinase 1 epsilon* and *delta* (*CK1ε* and *CK1δ*) phosphorylate PER and CRY, tagging them for polyubiquitylation by the E3 ubiquitin ligase complexes βTrCP1 and FBXL3, respectively, ultimately leading to their degradation by the 26S proteosome. In addition to phosphorylation mediated by the caseine kinase family, a role for GSK3-β signaling has also been established in flies [5]. Subsequent to PER and CRY phosphorylation, CLOCK/BMAL1 is released from repression, activating the forward limb of the 24 h cycle. The biochemical mechanisms involved in generating 24 h periodicity to CLOCK/BMAL1 activity remains an area of active investigation, although recent results suggest that posttranslational modification via phosphorylation of these factors may mediate the termination of their occupancy on promoters of the repressors [6, 7].

CLOCK and BMAL1 further drive expression of the orphan nuclear receptors, *Rev-erbα* and *Rorα*, which inhibit and activate *Bmal1* expression, respectively, by binding to the retinoic acid-related orphan receptor response element (RORE) within the *Bmal1* promoter, constituting a short-feedback loop [8, 9]. It is important to note that REV-ERBα and RORα are also key nutrient sensors (heme binds to

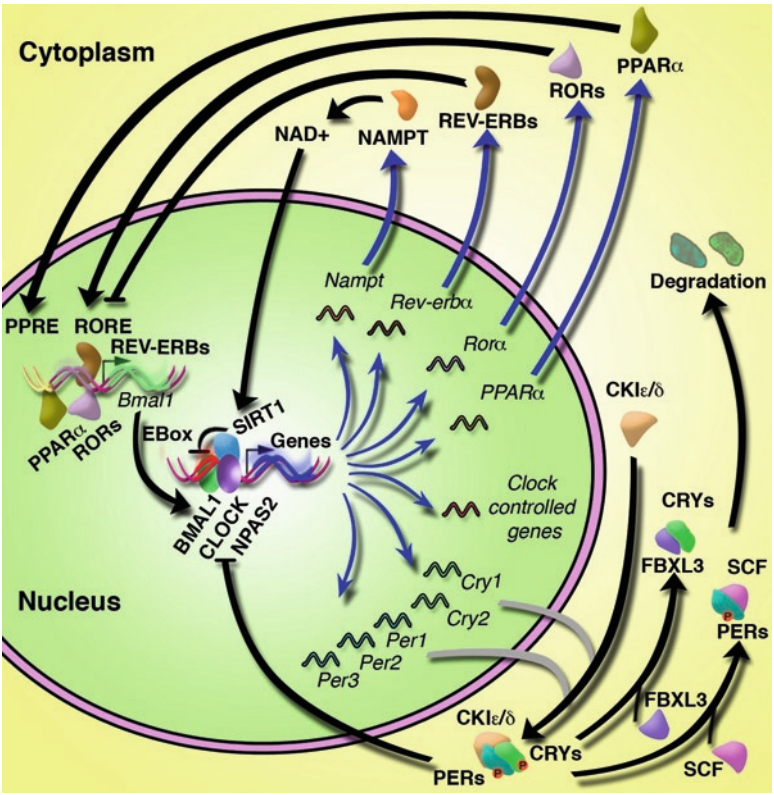

Fig. 1 The core molecular clock components (adapted from [32]). The core molecular clock machinery is encoded by a series of interlocking transcription–translation feedback loops that oscillates with a 24-h periodicity within both pacemaker neurons and within peripheral tissues. The positive limb of the clock is composed of the transcription factors CLOCK/NPAS2 and BMAL1, which heterodimerize and activate transcription of downstream clock target genes, including the period (*Per1*, *2*, and *3*) and cryptochrome (*Cry1* and *2*) genes, *Rev-erba*, *Rora*, and other clock-controlled genes. Upon translation, the PERs and CRYs heterodimerize, translocate back to the nucleus, and inhibit CLOCK/BMAL1 in a negative feedback loop. Multiple additional interlocking loops, including the nuclear receptors RORα and REV-ERBα which activate and inhibit *Bmal1*, respectively, are shown (please see text for further details)

REV-ERBα [10, 11], while cholesterol and oxysterols bind to RORα [12]), which provides clues as to a molecular link between metabolism and the circadian clock. This family of nuclear receptors is involved in the regulation of lipid and carbohydrate metabolism, as well as inflammation and thrombosis (reviewed in [13]).

Other nuclear receptors play an important role in the relationship between metabolism and circadian cycles. Indeed, the nuclear receptors known as peroxisome proliferator-activated receptor (PPARs) are lipid-activated transcription factors that have emerged as key regulators of lipid metabolism and inflammation [14]. For example, PPARγ displays circadian oscillation and controls *Bmal1* transcription

(detailed in part "From metabolism to circadian cycles"). Moreover, the PPARγ coactivator 1-α (PGC1α) also displays circadian oscillation in liver and skeletal muscle and upregulates the transcription of *Bmal1* and *Rev-erbα*. Since PGC1α levels are elevated in response to starvation, physical activity, and cold exposure, it will be important to learn whether *Pgc1α* is necessary to maintain circadian homeostasis under varying nutritional conditions.

Two additional molecular autoregulatory feedback loops link nutrient sensing and metabolism with the core circadian clock. The first of these involves the direct regulation of the rate-limiting enzyme in NAD^+ biosynthesis (nicotinamide phosphoribosyltransferase, *Nampt*) by the positive limb of the clock within peripheral tissues, including liver and white adipose tissue [15, 16]. Direct activation of *Nampt* by CLOCK and BMAL1 leads to elevated NAD^+ levels, increased activity of the NAD^+-dependent deacetylase SIRT1, and subsequently reduced CLOCK/BMAL1 activity, as SIRT1 is an inhibitor of the positive limb of the clock (Fig. 1). SIRT1 is also a key nutrient sensor that plays a critical role in the molecular integration of metabolism and circadian rhythms, as SIRT1 is also involved in a myriad of metabolic functions, including glucose and lipid metabolism, insulin secretion, and adipocyte differentiation (reviewed in [3]). A second mechanism involves circadian regulation via adenosine monophosphate-activated protein kinase (AMPK) signaling, a pathway activated by decreases in ATP production (and increases in AMP). AMPK modulates degradation of the core clock repressor, CRY1 [17]. Interestingly, AMPK has also been shown to modulate NAMPT activity, thus it is tempting to speculate that AMPK may also modulate circadian systems indirectly via activation of NAMPT [18].

Genetic mouse models have provided the opportunity to dissect the function of core clock genes in the generation and maintenance of circadian rhythms. *Bmal1* knockout mice [19] and mice with a dominant-negative *Clock* mutation [2] become arrhythmic in constant darkness. Of note, *Clock* knockout mice have normal locomotor activity rhythms due to developmental compensation by NPAS2 [20, 21]. Furthermore, *Per1*/*Per2* and *Cry1*/*Cry2* double knockout mice display a much more pronounced loss of circadian rhythmicity compared to the single mutant counterparts, consistent with either functional redundancy and/or developmental compensation [22–26]. Recent studies have also discovered that mutation of the F-box protein FBXL3 results in a period lengthening in mice [27, 28], and mice lacking PGC1α have abnormal diurnal locomotor activity rhythms and body temperature, along with altered expression of clock and metabolic genes [29]. While many of these early genetic studies focused on the role of clock genes in the regulation of circadian behavior, more recent studies have expanded the analyses of these mice to include their metabolic phenotypes, as discussed in section "Circadian Genes Involved in Metabolism Regulation". Further, the recent discovery that many nutrient-responsive factors, including the nuclear hormone receptors and the sirtuins, are key regulators of the clock have provided critical clues as to the molecular mechanisms linking metabolism and nutrient-sensing with the clock and sleep. Indeed, hormones and nutrients might directly modulate the sleep/wake and feeding/fasting cycles (detailed in sections "Neurophysiological Structures: Interconnection Between Circadian,

Sleep and Energy Centers" and "From Metabolism to Circadian Cycles"), and an important question is whether nutrient signaling per se may affect these cycles by modulating the core properties of the suprachiasmatic nucleus (SCN) pacemaker.

Peripheral Clocks: Regulation of Circadian Metabolism

Molecular analyses have revealed that the clock network is also widely expressed throughout nearly every tissue/cell type in vertebrates [30, 31]. In addition to the master clock in the SCN, independent circadian oscillators have been found in a number of peripheral tissues in mammals and can be maintained and self-sustained ex vivo in appropriate conditions. Gene expression profiling has shown that 3–20% of genes display a 24 h rhythmic expression, and a large proportion of these genes have a role in metabolic processes (for review [3]), including regulation of lipid and cholesterol biosynthesis, carbohydrate metabolism and transport, oxidative phosphorylation, and xenobiotic detoxification pathways (review [32, 33]). While the core clock machinery only directly regulates a small subset of these metabolic genes, oscillation of the nuclear receptors in metabolic tissues appears to indirectly regulate the expression of metabolic genes (for review [3]). Importantly, the period and amplitude of oscillation, as well as the level of expression of each of these metabolic genes, varies among different tissues, suggesting the importance of tissue-specific roles of peripheral clocks for normal cellular function. In this way, circadian patterns of metabolic gene expression may optimize the switch between daily anabolic and catabolic states corresponding with periods of feeding and fasting. For example, the cyclic expression of gastrointestinal tract enzymes may ensure that factors involved in nutrient absorption are expressed in anticipation of daily episodes of food ingestion, while adipose enzymes involved in fatty acid storage peak coincident with feeding. Moreover, components of gluconeogenesis, glycolysis, and fatty acid metabolism in the liver and a large portion of rhythmic genes in the muscle peak during the subjective night (in nocturnal rodents), coinciding with the peak of physical activity.

Thus, peripheral oscillators are cell-autonomous and tissue-specific, but the mechanisms involved in sustaining this synchrony within and between peripheral tissue clocks are still poorly understood. In addition, while the SCN is still considered the master clock, experimental genetic models suggest that the misalignment of local circadian oscillation among peripheral tissues or between peripheral tissue and SCN may contribute to cardiovascular and metabolic pathologies. For example, clock gene disruption targeted to the fat body in flies is sufficient to induce increased food consumption, decreased glycogen levels, and increased sensitivity to starvation [34]. At least in flies, these findings suggest involvement of a peripheral tissue clock in neural energy homeostasis [34]. A recent study reported that mice with a liver-specific deletion of *Bmal1* exhibited hypoglycemia during fasting, indicating a role for the liver clock in maintaining euglycemia during rest [35]. In addition, high-fat feeding (HFD), as well as mouse models of type 2 diabetes, alters

both circadian behavior and sleep [32]. Thus, identifying the signals that impact both central pacemaker neurons and peripheral clock oscillators which remains an area of intensive investigation.

From Circadian Disruption to Cardiometabolic Diseases

Impact of Sleep and Circadian Cycles on Metabolism: Clinical Evidence and Experimental Models

Impact of Sleep Quantity and Quality

Human studies. Epidemiological evidence has linked obesity and cardiometabolic disease (e.g. cardiovascular disease, type 2 diabetes) with both habitually short and long sleep. Numerous cross-sectional, as well as prospective clinical studies, have demonstrated that short-duration and poor-quality sleep predicts the development of type 2 diabetes and obesity after age, BMI and other variables are taken into account [36–41]. For example, short-term sleep duration (less than 6 h) in a large population of Japanese men has been associated with weight gain and development of obesity [42]. More alarming, such positive associations between short sleep duration and obesity have also been found in children [43, 44].

In addition to obesity, chronic short sleep duration is also strongly associated with cardiovascular disease and hypertension. Possible mechanisms linking sleep deprivation with cardiometabolic disease may include effects on glucose metabolism, appetitive behavior, and energy expenditure (reviewed previously [38, 39]). For example, healthy subjects who underwent six consecutive nights of sleep restricted to 4 h exhibited impaired insulin sensitivity following a glucose challenge. Furthermore, sleep deprivation results in a reduction of leptin and an increase in levels of the orexigenic hormone ghrelin, both of which may lead to increased appetite and altered energy expenditure.

Diseases related to changes in time and/or quality-sleep duration are also associated with metabolic disorders. For example, sleep apnea syndrome, a sleep disorder that is highly prevalent in metabolic syndrome [45], was proposed to cause clock gene dysfunction [46], and effective treatments of sleep apnea have been found to improve glucose metabolism and energy balance [40]. In addition, the circadian oscillation of leptin was found to be disrupted in narcoleptic patients, which may predispose them to weight gain [47]. A challenge for future investigation will be to discern the independent effects of circadian misalignment vs. sleep restriction (and hypoxia) on metabolic functions.

Animal studies. Experimental models using chronic sleep deprivation paradigms have strongly corroborated the impact of sleep loss in metabolism (reviewed in [48]). These studies have shown a decrease in blood levels of the satiety hormone leptin, which may contribute both to dysregulation of appetite and hepatic glucose metabo-

lism. Consistent with human studies, these works also demonstrated increased ghrelin levels and alteration in glucose utilization. However, in both human and rodent studies, it will be important to understand the extent to which increased autonomic tone contributes to metabolic dysfunction following sleep-deprivation.

Impact of Circadian Misalignment

Human studies. Gradual sleep loss, as well as extension of work during the night, may disrupt synchrony between the periods of sleep/activity with feeding/fasting and corresponding cycles of energy storage/utilization. Indeed, recent evidence has also demonstrated that chronic circadian disruption might also increase susceptibility to such disorders. Behavioral cycles are normally aligned with the light–dark cycle. However, the dysregulation of sleep and activity can result in misalignment of the central and peripheral oscillators and desynchronization of behavior, metabolic gene expression, and hormone release, thereby leading to adverse metabolic physiological consequences. Indeed, these misalignments might lead to obesity and to cardiovascular disease, as often observed in shift workers. For example, shift work is associated with a 1.6 and 3.0-fold increased risk of cardiovascular disease for 45–55 years old men and women, respectively [49]. An altered postprandial lipid excursion has also been reported in shift workers, thereby providing a partial explanation for the increased occurrence of cardiovascular disease [50]. Interestingly, the incidence of acute myocardial infarction is also significantly increased after the transition to daylight saving time, reinforcing the deleterious impact of chronobiologic rhythm disruption [51]. Recently, Sheer et al. demonstrated adverse cardiometabolic endpoints in human subjects who underwent forced misalignment of behavioral and circadian cycles, simulating the conditions of jet lag and shift work within a controlled clinical setting [52]. In this study, the behavioral cycle of the subjects was extended to 28 h, under dim light, with 14 h rest and fasting alternated with 14 h of wakefulness, interspersed with four evenly spaced and isocaloric meals. When subjects ate and slept approximately 12 h out of phase from their habitual times, circadian desynchrony decreased leptin levels and resulted in hyperglycemia and hyperinsulinemia. Thus, this study suggests that synchrony between behavioral and physiological rhythms is advantageous to maintain normal glucose metabolism in otherwise healthy persons.

Animal studies. The impact of chronobiology in the pathogenesis of obesity and its related disorders has been mainly substantiated by experimental genetic studies describing the role of clock genes in adipose tissue and other metabolic organs. Please refer to section “animal studies” for details.

Impact of Feeding Time

Human studies. Concerning the development of adiposity, there are several clues to suggest that disruption of either behavioral or genetic aspects of circadian synchrony

may contribute to dymetabolic states. Indeed, several clinical studies have highlighted the importance of feeding time in a society in which people are inclined to eat at irregular times in the sleep-wake cycle (for review [53]). Conversely, regularity of food intake improves postprandial thermogenesis and reduces energy intake [54]. High-energy intake in the evening and/or skipping breakfast has been associated with the development of obesity (for review [53]). Curiously, individuals diagnosed with night eating syndrome appear to have greater propensity towards obesity.

Animal studies. Interestingly, mice fed a HFD consumed nearly all of the extra calories during the 12-h light phase, demonstrating a desynchronizing effect of HFD on the normal feeding rhythm [55]. A plausible hypothesis is that consuming calories at the incorrect time in the light–dark cycle (i.e., rest period) exacerbates the obesogenic effects of HF caloric intake due to desynchronization of various behavioral, hormonal, and molecular rhythms involved in maintaining energy balance.

Interestingly, genetically obese animals are resistant to weight gain when feeding is restricted to the active (dark) phase. In agreement with these observations, recent evidence demonstrated that circadian timing of food intake contributes to weight gain [56]. Indeed, mice fed with a HFD only during the 12-h light phase gained significantly more weight compared to isocalorically fed mice which were provided food only during the 12-h dark phase [56]. As expected, food intake during the normal activity phase prevents obesity and circadian desynchrony in a rat model of night work, based on daily 8-h activity schedules during the resting phase [56].

Further studies are necessary to understand how the timing of food intake impacts energy constancy. Interestingly, a study demonstrated that treatment with an antagonist of T-type calcium channel, which is involved in sleep–wake regulation, improved HFD-induced alterations, including a decrease in inactive phase activity, core body temperature, feeding, and adiposity [57]. Taken together, these observations (largely based on animal studies) raise important questions concerning the impact of circadian misalignment and clock gene disruption on obesity and its metabolic complications and suggest avenues for future investigation in human subjects. How does time of feeding affect circadian systems from the physiological to molecular level? Understanding this question has implications for public health, since overnutrition and altered rest–activity behavior are common in modern society, and both factors have been implicated in the pathogenesis of metabolic syndrome and cardiovascular disease.

Clock Genes Regulate Both Sleep and Metabolism: Genetic Evidence in Human and Animal Models

Circadian Genes Involved in Sleep/Wake Phenotypes

Human studies. In addition to environmental sleep disruption (e.g., shift work disorders), genetic polymorphisms in several clock genes have also been linked to sleep phenotypes. For example, an advanced sleep phase has been associated with polymorphism of the human *Per1* gene [58] and with missense mutations of *Per2*

[59] and *CKε/δ* [60, 61]. Mutations in several clock genes have also been identified in the familial (monogenic) sleep disorders in man, but it is not known whether these increase susceptibility to metabolic disorders [60–62].

Animal studies. The *Clock*$^{\Delta 19}$ mutant mouse was produced in a deliberate chemical mutagenesis screen designed to identify the genes controlling mammalian circadian locomotor activity [1, 63]. Interestingly, mice homozygous for the *Clock* mutation (on a C57Bl/6J background) sleep approximately 2 h less than wild-type mice in regular 12:12 light:dark conditions and present a smaller amount of rapid eye movement (REM) sleep rebound during 24 h recovery following sleep deprivation [64]. Since that time, there have been major advances in understanding the molecular basis of the clock network (described in section "The Core Clock Molecular Network: The Basis of Circadian Rhythms"), which is now viewed as a centerpiece for the generation of the 24 h rhythms of sleep propensity. Among the other clock genes described above, genetic animal models with *Cry* or *Bmal1* gene deletion also present sleep phenotypes. For example, mice deficient in *Cry1* and *Cry2* showed altered sleep structure, including increases in non-REM time, consolidation, and EEG delta power, in addition to their disrupted circadian period [65, 66]. *Bmal1* is also believed to be involved in the generation of sleep, as *Bmal1* mutant mice display increases in total sleep time, sleep fragmentation and EEG delta power under baseline conditions, and an attenuated compensatory response to acute sleep deprivation [67].

It is important to note that the circadian system is not the only mechanism regulating sleep. The wake-dependent homeostatic sleep process, whereby sleep pressure increases during wake and dissipates during sleep, represents a principal process controlling sleep (for review, see [68, 69]). A better understanding of the neurobiological links between sleep, energetics, and metabolism will likely emerge as the homeostatic process becomes more clearly defined at the molecular level.

Circadian Genes Involved in Metabolism Regulation

Human studies. Polymorphisms in several clock genes have been linked to obesity or to other features of the metabolic syndrome (for review, see [32]). In small sample populations, polymorphisms in the *Clock* gene have been correlated with predisposition to obesity, and two *Bmal1* haplotypes are associated with type 2 diabetes and hypertension. Polymorphisms within other clock core genes (i.e., *Per2* and *Npas2*) have also been associated with hypertension and high fasting blood glucose in studies of similar sample size. Interestingly, a rare variant in *Nampt* (*Visfatin/Pbef1*), which is involved in a negative clock feedback loop, is associated with protection from obesity.

In addition to the clock gene machinery, several genome-wide association studies recently discovered that melatonin, a hormone implicated in seasonal and circadian rhythms, and its receptor melatonin 1B receptor gene (*mtnr1b*), may be important in the regulation of mammalian glucose levels [70]. Interestingly, polymorphism in *Cry2* has also recently emerged as a genetic factor involved in fasting glucose regulation in large-scale association studies [71]. Taken together, these studies suggest

that disruption of circadian systems may contribute to human metabolic syndrome and cardiovascular complications, either directly at the level of altered clock gene expression, or indirectly through effects on melatonin.

Animal studies. The discovery that *Clock*$^{\Delta 19}$ mutant animals develop hyperglycemia, hyperlipidemia, hepatic steatosis, and increased susceptibility to diet-induced obesity has provided a new entrée into experimental studies to dissect the mechanistic linkages between circadian and metabolic systems [72]. The feeding rhythm in these mice is damped, with increased food intake during the day, and, in addition, these mice have significantly increased food intake overall. HF feeding studies revealed exaggerated weight gain of *Clock*$^{\Delta 19}$ mutant mice, and DEXA scanning and fat pad weight both demonstrated significant increases in fat and lean mass relative to controls following HF feeding. It is likely that the obese phenotype results, at least in part, from altered rhythms of neuropeptides in the hypothalamus, as ghrelin, cocaine- and amphetamine-regulated transcript (CART), and orexin are all expressed at constitutively low-levels in the *Clock*$^{\Delta 19}$ mutant mice. In addition, the anorectic neuropeptide pro-opiomelanocortin (*POMC*) was decreased throughout the entire LD cycle in hypothalami of young *Clock*$^{\Delta 19}$ mutant prior to the onset of weight gain and overt diabetes, consistent with a deficit in the central homeostatic regulation of weight constancy. Disruption of other circadian clock genes also leads to metabolic alterations. For example, gene disruption in *Bmal1* induces an abnormal metabolic phenotype characterized by impaired gluconeogenesis, hyperleptinemia, glucose intolerance, and dyslipidemia [35, 73, 74]. In addition, *Per2* knockout mice developed increased weight gain on HFD [75]. Conversely, mice deficient in the circadian deadenylase nocturin remained lean and resistant to hepatic steatosis when fed a HFD despite equivalent caloric intake, similar metabolic rates, and reduced activity compared with control mice [76].

In addition to changes in sleep and circadian cycles affecting metabolism, alterations in metabolism are also able to entrain central and/or peripheral clocks, thereby resulting in changes to the rhythms of sleep/wakefulness, fasting/feeding, and hormonal secretion and energy metabolism. Indeed, nutrient and hormonal cues may also affect the period and phase characteristics of the master clock neurons, although little is known about how metabolic signals are communicated to the SCN. In addition, it is still unclear whether the food-entrainment pathway regulates circadian behavior directly or indirectly through other brain structures.

Neurophysiological Structures: Interconnection Between Circadian, Sleep and Energy Centers

Circadian and Sleep Centers

The past several decades have witnessed enormous outgrowths of understanding regarding the brain centers important in the regulation of circadian rhythms, sleep, and metabolism (Fig. 2). One of the early seminal experiments to show that

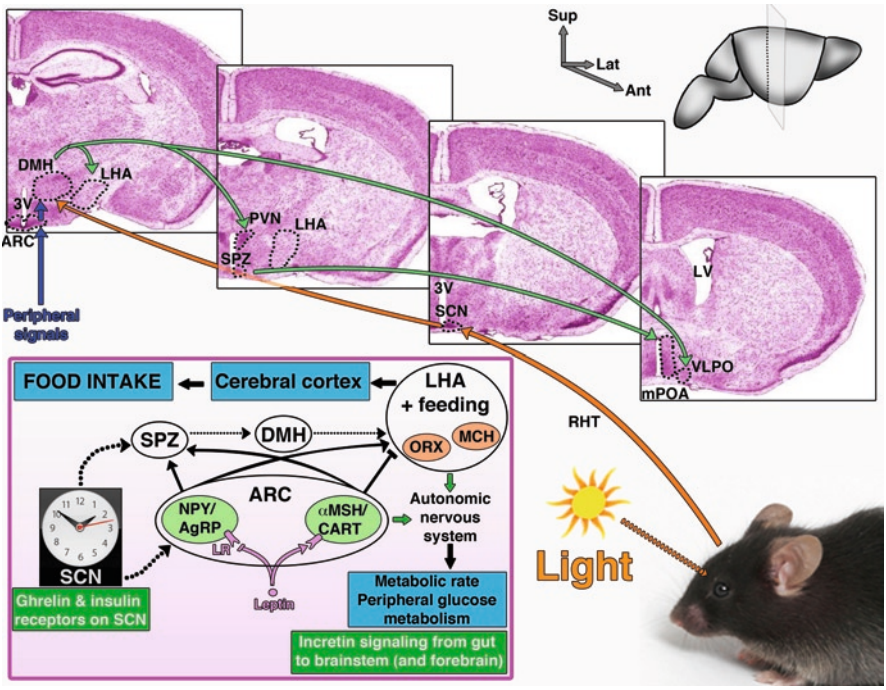

Fig. 2 Neural pathways linking circadian and metabolic systems (adapted from [3]). Light is the predominant environmental cue that is transmitted from the eyes via the retinohypothalamic tract (RHT) to the SCN. Projections from the SCN extend toward the SPZ and then onward from the SPZ to the dorsomedial hypothalamus (DMH). The SPZ and DMH also project toward the medial preoptic area (mPOA) and the VLPO, two relay regions of the hypothalamus that may be served to integrate circadian and wakefulness signals. The DMH has emerged as an important site in the activity response to food (the food-entrainable oscillator), although this finding remains controversial. The DMH has many outputs to other regions of the brain, including the LHA, which controls circadian regulation of the sleep/wakefulness and fasting/feeding cycles. *Inset*: The LHA also receives neuroendocrine input from the arcuate (ARC) neurons producing anorexigenic and orexigenic neuropeptides. The hormone leptin produced by adipose tissue activates the production of anorexigenic neuropeptides such as αMSH/CART, which in turn blocks production of the orexigenic peptides orexin (ORX) and MCH in the LHA. In the absence of leptin, orexigenic neurons in the ARC produce the neuropeptides NPY/AgRP that stimulate hunger and decreased energy expenditure via signaling to the LHA. In addition, insulin, ghrelin, and other incretins may also influence circadian behavioral rhythms through direct effects on SCN or indirectly through actions within other regions of midbrain and brainstem. Arrows in inset indicate functional links. *3V* third ventricle; *LV* lateral ventricle

circadian period length was determined by the SCN involved in the restoration of normal circadian locomotor activity in SCN-lesioned hampsters by transplantation of a wild-type SCN from another animal [77]. Of note, these activity rhythms were recovered despite the lack of direct neural connections between the grafted SCN and the host brain, suggesting that a diffusible secreted factor, such as transforming growth factor-a, prokineticin-2, gamma-aminobutyric acid, or vasopressin, might

be important for the generation of at least some circadian rhythms. The SCN has numerous cellular and anatomic projections to hypothalamic centers involved in the regulation of wakefulness, activity, and feeding (reviewed in [68, 78]). In particular, the largest output of projections is directed toward the subparaventricular zone (SPZ); the ventral SPZ regulates circadian rhythms of locomotor activity and sleep/wakefulness, while the dorsal SPZ regulates circadian rhythms of body temperature. Both the SCN and SPZ also project to the dorsomedial nucleus of the hypothalamus (DMH), which has been implicated in circadian rhythms of locomotor activity, sleep/wakefulness, corticosteroid secretion, and feeding. In turn, the DMH projects to other brain centers involved in the regulation of sleep (ventrolateral preoptic nucleus, VLPO), corticosteroid release (the paraventricular nucleus, PVN), and wakefulness/feeding (the lateral hypothalamus, LHA). Of note, the DMH has been implicated in the ability of an organism to be entrained by food, although this finding remains controversial (see section "From Metabolism to Circadian Cycles"). Sleep recording in sham and SCN-lesioned mice under baseline conditions and following sleep deprivation has also established that the SCN plays a central role in the regulation of sleep and wakefulness beyond just the timing of vigilance states [79]. Recent functional magnetic resonance imaging experiments in humans with extreme chronotypes also demonstrate that vigilance state in the evening was associated with higher activity in evening than morning chronotypes in a region of the suprachiasmatic area including the circadian master clock [80]. This activity may be modulated by homeostatic sleep pressure [80].

Link Between Energy Centers with Circadian and Sleep/Wakefulness Centers

In addition to brain centers regulating circadian locomotor activity and sleep/wakefulness, recent advances have been made toward understanding the overlap between these circadian centers with those involved in energy balance and feeding behavior (Fig. 2). Importantly, a landmark breakthrough in our molecular understanding of the hypothalamic control of appetite regulation and energy balance came in 1994 with the positional cloning of leptin, a secreted adipocyte-derived factor, and subsequent cloning and localization of its receptor within various regions of the hypothalamus, including the arcute nucleus (ARC), DMH, and VMH, all regions previously implicated in regulation of satiety (reviewed in [68, 81]). Of note, leptin is secreted in proportion to the fat mass, and its levels display circadian rhythmicity in addition to responding to nutrient status. Thus, these findings have provided critical insight into how signals from the periphery may translate the nutritional status of the organism to appetite-regulating regions of the hypothalamus in a circadian-dependent fashion.

Following the initial discovery of leptin and its receptor, came the discovery of the melanocortin system as downstream of leptin signaling (reviewed in [68, 81]). Leptin activates the POMC and CART-expressing neurons within the ARC to release α-melanocyte-stimulating hormone (α-MSH), which then activates the

melanocortin receptor subtype 4 (MC4), leading to inhibition of food intake and increased energy expenditure. At the same time, leptin inhibits the neuropeptide Y (NPY) and agouti-related protein (AgRP)-expressing neurons within the ARC, thereby in effect blocking the ability of α-MSH to act on its MC4 receptor via release of AgRP and inhibiting the POMC/CART-expressing neurons via release of small inhibitor amino acid neurotransmitter γ-aminobutyric acid. Thus, during fasting, when leptin levels are low, the orexigenic neuropeptides (NPY and AgRP) lead to increased appetite and decreased energy expenditure, while during feeding, leptin acts to suppress appetite via activation of POMC/CART.

These orexigenic and anorexigenic neuropeptides further project to additional centers within the brain involved in feeding behavior, including the LHA, which makes the orexigenic melanin-concentrating hormone (MCH) and the orexins A and B (reviewed in [82]). Importantly, orexins A and B display circadian rhythmicity, are induced by fasting, and play a critical link in the regulation of sleep–wake rhythms, as their neurons also project to regions within both the cortex and brainstem which regulate arousal and autonomic function. Disruption of orexin and its receptor result in narcolepsy, which is consistent with its role in the regulation of the sleep–wake axis. Finally, neurons from the ARC also project to the dopaminergic-reward centers of the midbrain, including the ventral tegmental area (VTA), suggesting a direct link between the appetite-regulating regions of the brain with those important in regulating reward in response to food and drugs [83, 84]. The extensive anatomic projections and synaptic relays between the various brain centers involved in circadian rhythmicity, sleep/wakefulness, and feeding indicate that the brain has evolved to be able to rapidly coordinate and allow extensive cross-talk between these centers, thereby allowing the organism to most efficiently adapt to daily changes in its environment.

From Metabolism to Circadian Cycles

As described above, the master pacemaker has anatomic connections with centers coordinating activity behavior, sleep, appetite, and energetics, suggesting that targeting one of these centers would impact the others. Indeed, feeding behavior in particular plays an essential role in coordinating the circadian rhythms of sleep and activity. Indeed, it is well known that food restriction to the normal rest period in rodents also induces a burst of food anticipatory activity (FAA). While lesioning of the dorsomedial nucleus has been shown to alter FAA [85–87], there remains controversy regarding the involvement of circadian oscillators in FAA since the FAA behavior persists in *Bmal1* nullizygous mice [85, 88]. Recent data also suggests the involvement of the melanocortin signaling pathway in FAA [89]. Further, the FAA may constitute a metabolic oscillator responsive to peripheral neural or circulating signals elicited by food ingestion. Resolution of the precise stimuli and neural pathways involved in FAA, as well as understanding the involvement of nutrient signaling pathways which may affect core properties of the SCN pacemaker, remain important questions for the future.

As mentioned earlier, diet-induced obesity per se alters circadian behavioral and molecular rhythms in C57BL/6J mice [55]. Indeed, HFD in mice leads to increased daytime activity, a lengthened period of the locomotor activity rhythm, and alterations in the expression and cycling of canonical circadian clock genes, nuclear receptors that regulate clock transcription factors, and clock-controlled genes involved in fuel utilization in the hypothalamus, liver, and adipose tissue [55]. Conversely, it has also been demonstrated that caloric restriction induces phase advances in rat behavioral and physiological circadian rhythms (for review [90]). Indeed, prolonged fasting advances the phase of free-running rhythms such as wheel-running and body temperature. Oscillatory expression of clock genes and neuropeptides in the mouse SCN are also altered by hypocaloric feeding, supporting the hypothesis that calorie restriction has effects within the SCN clock (for review [90]).

Changes in dietary nutrient composition, calorie content, or in food availability play an important role in the regulation of circadian behavior and physiology, although the mechanisms of such regulations are still unclear. For instance, availability of food, in addition to other environmental factors such as day length and temperature, might strongly regulate the timing of torpor induction. Torpor is a state of "inactivity" during mammalian hibernation, which is homologous to sleep as demonstrated by numerous EEG studies (for review [91]). Indeed, there is a switch from carbohydrate- to fat-based metabolism during torpor. Most metabolic processes seem to be halted or slowed considerably during deep torpor. Prior to hibernation, an animal dramatically increases its food intake and stores energy in the form of fat. During the low-temperature torpor phases, the burning of fat results in the accumulation of acetyl-CoA, which becomes converted into heat (by uncoupling proteins) and energy upon the transient activation of mitochondrial oxidative phosphorylation during brief interbout arousals. The signals that trigger the periodic arousals from torpor have not yet been elucidated. However, the studies of hibernating species allowed postulating that it is the fluctuating levels of these key metabolites that control the transitions between torpor and interbout arousal. Of note, the SCN keeps a relatively high metabolic activity compared to nearly every other brain structures in torpor, as demonstrated namely by uptake of [14C] 2-deoxyglucose measurement (for review, see [91]).

On the other hand, sleep change affects many aspects of our physiology and behavior, from immunity to hormonal regulation. Indeed, brain metabolism itself changes depending on the sleep state; it is low in NREM sleep but high in REM sleep (for review [92, 93]). Whole-genome transcriptomic studies have revealed a differential expression of many genes between brains of sleeping and awake animals. These changes occur mainly in the cerebral cortex, cerebellum, and hypothalamus. The transcripts that are the most consistently increased during waking and short-term sleep deprivation relative to sleep genes include genes involved in energy metabolism, including those coding for mitochondrial proteins, glucose transporters, and proteins related to glycogen metabolism (for review [92, 93]). Their upregulation has been proposed to be a mechanism by which the brain responds to the high-energy requirements of wakefulness (for review [92, 93]). Conversely, the transcripts with increased expression during sleep appear to be mainly involved in protein synthesis and lipid metabolism (for review [92, 93]).

Fatty Acids/Lipids

Studies performed more than 20 years ago by Brody and colleagues were the first to identify interactions between fatty acids and circadian oscillator function. Analyses in a fatty acid-requiring strain of *Neurospora* indicated a relationship between the period length of the spore-forming rhythm and unsaturated fatty acid concentration of medium. The period lengthening effects of unsaturated fat were reversed by the addition of saturated fat, indicating a specific correlation between lipid signals and oscillator properties, although the metabolic pathways accounting for these effects at a mechanistic level have not been uncovered [94].

During recent years, studies undertaken in rodents demonstrated that fatty acids can relay the body's nutritional status to the hypothalamic energy center (the arcuate nucleus), thereby controlling feeding behavior (for review [95]). Long chain fatty acids cross the blood brain barrier (BBB) mainly by simple diffusion in the unbound form or via delivery by chylomicrons or other circulating lipoproteins. Cellular accumulation of long-chain fatty acyl-CoA, as well as manipulation of enzymes of the fatty acid synthesis pathway that result in elevated malonyl-CoA, lead to inhibition of food intake (for review [95, 96]). Of note, in addition to hypophagia, mice presenting a deletion of fatty acid synthase in hypothalamus (and islets) showed significantly increased locomotor activity, particularly during the dark phase [97], suggesting that fatty acids might act centrally to control daily behavior.

In addition to the regulation of feeding behavior, lipid metabolism may also play a role in sleep, as HFD fed to female C57BL/6J mice increased their sleep time [98]. Further, QTL analysis identified Acads, the short-chain acyl-coenzyme A dehydrogenase involved in fatty acid β-oxidation, as linked to theta frequency, which is prominent during REM sleep; however the mechanism by which its deficiency can slow down the peak theta frequency remains unclear (for review [93]). Finally, forward molecular and reverse genetic approaches have shown that *rar*-β, the gene encoding retinoic acid receptor-β, is important for determining the contribution of delta activity to the EEG during NREM sleep (for review [93]).

Fatty acids have also been described as interfering in peripheral circadian physiology, especially within the vascular tissue. Conditional deletion of PPARγ, the rhythmically expressed lipid receptor that directly regulates *Bmal1* transcription, within vascular tissue results in abnormalities in blood pressure and heart rate in parallel with a reduction of diurnal variation in the sympathetic nerve activity [99]. Furthermore, vascular PPARγ exhibits a robust cyclic expression, whose rhythmic phase may be reset by changes in feeding time as well as changes in the photoperiod [99]. Thus, the temporal environment may be integrated within the heart by PPARγ. Consistent with this, PPARγ agonists were found to shift the circadian fluctuation of blood pressure in patients with type 2 diabetes, indicating that vasculoprotective actions of thiazolidinediones may in part involve effects on the clock transcription network [100]). Emerging clinical evidence has also uncovered unique actions of the PPARα agonist fenofibrate in the circadian control of blood pressure and heart rate in diabetic subjects [101–103].

In turn, the circadian clock also controls lipid metabolism. For instance, the clock induces an ultradian rhythm in the expression of genes involved in the unfolded protein response, thereby controlling rhythmic regulation of hepatic lipid metabolism [104].

Amino Acids

Caloric restriction, amino acid imbalance, and activation of the target in rapamycin pathway *increase* life span in evolutionarily diverse organisms including mammals [105, 106]. While the mechanisms involved are still not completely understood, it has been postulated that the biological clock could be an important mediator of longevity in calorically restricted animals (for review [107]). Interestingly, many amino acids exhibit significant circadian rhythmicity in both mice and humans. For example, glutamine, threonine, proline, valine, phenylalanine, methionine, isoleucine, leucine, and tryptophan peak around midnight, as demonstrated by mass spectrometry analysis of mouse blood samples [108]; however it is unclear how exactly amino acids may by regulated by the clock. Studies show that diets low in proteins increase food intake and, conversely, diets high in protein decrease food intake, potentially implicating the CNS in amino acid sensing (for review [109]). Indeed, central administration of leucine, a branched-chain amino acid, inhibits food intake, whereas valine has no effect, illustrating the importance of this particular amino acid. The target of rapamycin pathway appears to be important in CNS amino acid sensing, and its activity is regulated by feeding/fasting states (for review [109]). Lastly, the activation of the target of rapamycin pathway in SCN might also potentially be involved in light entrainment process [110].

Carbohydrates

Mammalian glucose metabolism *displays* pronounced diurnal variation across the light–dark cycle, with alternating cycles of gluconeogenesis and glycogen synthesis that are coordinated with the rest–activity cycle (reviewed in [81]). In addition, glucose availability has recently been described to control circadian rhythmicity in fibroblasts [17]. Intriguingly, in addition to liver, a small amount of glycogen is also synthesized and stored in brain astrocytes. Fifteen years ago, it had been hypothesized that these stores might be used/depleted during wakefulness and restored during sleep, which has been reinforced by numerous observations (for review [111]).

Cellular Energy Status

Pacemakers in peripheral organs, such as the liver, are reset by food availability. AMPK, an enzyme that responds to nutrient availability, is involved in this

entrainment. Indeed, activation of AMPK correlated with phase advances in mice after treatment with metformin, an AMPK-targeting antihyperglycemic biguanide [112]. Recently, Lamia et al showed that AMPK directly phosphorylates the core clock protein cryptochrome 1 (CRY1), thereby marking it for degradation [17]. AMPK is activated upon its phosphorylation by protein kinases such as liver kinase B1 (LKB1) or calcium-calmodulin-dependent protein kinase β, which sense the AMP/ATP ratio, a direct readout of the cell's metabolic state [17]. In addition to these regulatory functions in peripheral tissues, AMPK has been hypothesized to play the role of energy sensor in the hypothalamus. Both pharmacological approaches targeting AMPK either directly or indirectly through modulation of central fatty acid metabolism and central injection of adenovirus, consistently demonstrated that AMPK activity is strongly involved in regulation of feeding behavior (for review [113]). Thus, AMPK might also serve as a molecular sensor to shift the brain from energy consuming synthetic processes that occur during sleep to catabolic energy-producing processes that occur during wakefulness (for review [111]) and might contribute to sleep homeostasis [114].

Other components of the adenosine metabolic pathway have been proposed to couple the metabolic and circadian cycles. 5′-AMP, which is elevated in the blood of DD mice, is able to induce torpor [115]. In addition, intracellular adenosine 3′, 5′-monophosphate (cAMP) oscillates in the mouse SCN [116], as well as in other brain areas [117], although it is important to note that mice are nonhibernating mammals. cAMP sustains the transcriptional loop of the SCN, determining canonical pacemaker properties of amplitude, phase, and period [116]. Brain cAMP levels might also be regulated during sleep deprivation [118] and regulate sleep/wake cycles [119].

Another signal linking metabolic and circadian systems has recently been described by several groups. The ratio of oxidized nicotinamide adenine dinucleotide phosphate (NAD^+) to its reduced form (NADH) is related to feeding/fasting state and may entrain peripheral clocks [15, 16]. Importantly, NAD^+ biosynthesis varies across the light–dark cycle, suggesting that NAD^+ functions as an oscillating metabolite linking circadian and metabolic cycles [16]. The major node regulating NAD^+ biosynthesis involves the rate-limiting enzyme nicotinamide phosphoribosyl transferase (*Nampt*), which oscillates in a circadian manner and is directly regulated at the transcriptional level by CLOCK/BMAL1. Alterations in *Nampt*/NAD^+ modulate the nutrient-responsive deacetylase SIRT1, which plays an important role in the regulation of glucose and lipid metabolism, insulin secretion, the inflammatory response, and the circadian clock. This pathway is particularly intriguing in light of the fact that NAMPT and SIRT1 are regulated not only by the clock, but also by the nutritional status of the organism. For example, AMPK is able to modulate NAD^+ metabolism and SIRT1 activity [18]. In addition, *Nampt* is upregulated in response to glucose restriction in skeletal muscle in an AMPK-dependent manner [18, 120]. Thus, regulation of the clock by NAD^+ and SIRT1 allows for coordination of the core clock machinery with the daily cycles of fasting/feeding and rest/activity. It has also recently been demonstrated that NAMPT is secreted and is present in the circulation, though it is not yet known whether extracellular NAMPT is regulated in a circadian manner, thereby influencing downstream processes on a

systemic level. Interestingly, NAMPT concentration in cerebrospinal fluid is decreased with increasing body fat, but further investigation will be necessary to clarify this link in humans [121].

Hormonal Mediators

Central insulin. Strong evidence from human studies demonstrates rhythmic variation in glucose tolerance and insulin action across the day. Both insulin secretion and sensitivity are decreased in the evening (for review [81, 122]). Insulin may also modulate circadian behavior, as insulin is able to reach the brain via a saturable transporter across the BBB. Mice with a brain-specific insulin receptor (IR) deficiency and mouse models with inducible IR inactivation demonstrated that central insulin action plays an important role in the regulation of food intake, as well as peripheral glucose, and fat metabolism. These effects are mediated through phosphatidylinositol 3 kinase (PI3 kinase) and mitogen-activated protein kinase (MAPK) cascades. Activation of the PI3 kinase results in activation of protein kinase B/Akt and phosphorylation of FOXO, which is of critical importance for maintenance of energy homeostasis by the CNS. Insulin might also be involved in sleep/wake behavior [119]. Lastly, a genome-wide small interfering RNA screen in a human cellular clock model demonstrate that among the numerous pathways interconnected with clocks, the insulin-signaling pathway was overrepresented [123]. Thus, it will be interesting to examine whether insulin acts on SCN to control circadian behavior.

Central leptin. The fall of leptin that occurs rapidly in response to fasting also evokes profound changes in energy balance via the hypothalamus. Like insulin, leptin is also involved in the control of feeding behavior. Leptin from the periphery is transported into the brain, binds to its receptor in the hypothalamus, and activates janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3), leading to suppression of "orexigenic peptides" (e.g., NPY and AgRP), and elevation of "anorexigenic peptides" (e.g., POMC and CART) [124], thereby curtailing food intake. In the common form of obesity, resistance to leptin has been ascribed to diminished transport of leptin across the BBB and to elevated hypothalamic levels of SOCS3 and endoplasmic reticulum (ER) stress, which inhibit leptin signaling [125–127]. Furthermore, AMPK mediates both leptin's anorexigenic effect, as well as adiponectin's orexigenic effects [128, 129]. In addition, the study of sleep in several mouse models of obesity and diabetes has demonstrated leptin's involvement in sleep architecture. For example, *ob/ob* mice, a genetic model of leptin deficiency, has an elevated number of arousals from sleep [130], while *db/db* mice (which harbor a mutation in a particular isoform of the long form of leptin receptor), exhibits increased overall sleep time, a dramatic increase in sleep fragmentation, attenuated diurnal rhythmicity in REM sleep and non-REM EEG delta power, and a decrease in the compensatory response to acute sleep deprivation [131].

Taken together, these data suggest that leptin resistance might be a potential link between HF feeding, alterations of diurnal behavior, and sleep rhythms.

Central inflammation. Adipokines and cytokines are also able to cross the BBB and may modulate sleep. For instance, interleukin-6 and tumor necrosis factor-α in plasma, which are increased in obesity, may also impair circadian clock gene oscillations and promote sleep (for review [32]). Hypothalamic inflammation may also cause disrupted feeding behavior and obesity [132, 133], although a more "acute" central inflammation may preferentially lead to anorectic behavior. Thus, it is tempting to speculate that some inflammatory factors might directly target the SCN. It is known that, for instance, intracerebroventricular injection of recombinant receptor-activator of NF-κB ligand (RANKL) triggers c-Fos activation in the SCN [134]. Mice intraperitoneally injected with LPS exhibited abnormal diurnal activity, while deleting RANKL receptor in brain abolish this phenomenon [134].

Obesity and nutrient overflow result in conditions that increase demand on the ER in metabolic tissues including liver, adipocytes, and pancreas, resulting in a persistent inflammatory state [135], as well as hypothalamus, thereby altering feeding behavior. Interestingly, a group of transcripts strongly upregulated during wakefulness code for proteins involved in the ER stress response, chaperones, and heat-shock proteins. During waking, the expression of proteins implicated in stress responses increases, suggesting that absence of sleep could be a stress for brain cells (for review [92, 93]). For instance, the ER chaperone protein BiP, a key protein involved in the ER stress response, is expressed in a circadian manner [104, 136]. Thus, disrupted synchrony of stress response, gene expression may potentially alter circadian and sleep disturbances.

Lipids, Endotoxins and Hormones from the Gut

Several gut satiety factors produced in response to fat ingestion might also contribute to food entrainment. For instance, duodenal infusion of fat stimulates small intestinal mucosal cells to produce the lipid messenger oleoylethanolamide enabling CD36-mediated uptake of dietary oleic acid and thus promoting satiety [137]. Interestingly, this factor shows diurnal variation in cerebrospinal fluid of rats [138]. Moreover, plasma lipid N-acylphosphatidylethanolamines (NAPEs) are also secreted into circulation from the small intestine in response to ingested fat [139]. Interestingly, systemic administration of circulating NAPE decreases food intake and locomotor activity in rats [139]. Furthermore, mice fed with a HFD also display enhanced metabolic endotoxemia induced by the death of gram-negative bacteria within gut that participate in the occurrence of metabolic disorders [140]. The endotoxin LPS exhibits a diurnal variation that is disrupted by HF feeding [140]. As described above, inflammatory molecules such as LPS may induce sleep, suppress biological clock genes, and promote anorexia. Among gastrointestinal hormones, incretins also show circadian variation [141]. Furthermore, the reversibility of insulin resistance observed after biliopancreatic diversion may be related to an improvement in the circadian

control or pattern of incretin production [141]. Ghrelin, a stomach-derived hormone, which participates in meal initiation, also displays circadian rhythms, and the amplitude of its rhythm is reduced in obesity [142, 143]. Indeed in healthy subjects, high levels of ghrelin are detected in the early morning, when eating is precluded by sleep; however, this peak is not present in obese subjects [143]. Of interest, in addition to its orexigenic role, ghrelin has been shown to stimulate locomotor activity in anticipation of meals [144] and to increase slow-wave sleep [145], thereby playing an important role in the control of circadian behavior and perhaps even sleep architecture.

Conclusions

During recent years, much progress has been made in the dissection of the neurobehavioral basis of feeding, sleep, and circadian timing. In addition, numerous epidemiological as well as experimental genetic studies have demonstrated that metabolic networks are under extensive circadian control and that alterations in the circadian clock promote the development of obesity and metabolic diseases. However, further investigation will be necessary to understand on a molecular level how perturbations of the internal clock system and sleep constitute risk factors for metabolic disorders. Finally, it will be important to determine how nutrient affects the circadian system and the molecular control of behavior. Efforts to dissect the molecular mediators that coordinate circadian, metabolic, and cardiovascular systems may ultimately lead to both improved therapeutics and preventive interventions.

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