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Review

The emerging roles of lipids in circadian control ☆

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ABSTRACT

Lipids play vital roles in a wide variety of cellular functions. They act as structural components in cell membranes, serve as a major form of energy storage, and function as key signaling molecules. Mounting evidence points towards a tight interplay between lipids and circadian clocks. In mammals, circadian clocks regulate the daily physiology and metabolism, and disruption of circadian rhythmicity is associated with altered lipid homeostasis and pathologies such as fatty liver and obesity. Concomitantly, emerging evidence suggest that lipids are embedded within the core clock circuitry and participate in circadian control. Recent advances in lipidomics methodologies and their application in chronobiology studies have shed new light on the cross talk between circadian clocks and lipid homeostasis. We review herein the latest literature related to the involvement of lipids in circadian clock's function and highlight the contribution of circadian lipidomics studies to our understanding of circadian rhythmicity and lipid homeostasis. This article is part of a Special Issue entitled Brain Lipids.

1. Circadian rhythms—introduction

Light-sensitive organisms from cyanobacteria to humans harbor time-measuring devices, known as circadian clocks, which allow them to anticipate daytime and hence regulate their physiology and behavior in a proactive manner. Circadian clocks ("circa diem" means "approximately a day") measure about 24 h and therefore need to be periodically readjusted to the geophysical time. The most dominant environmental *Zeitgeber* (time giver) for the phase entrainment of circadian oscillators is dark–light cycles [1].

The mammalian circadian clock is structured in a hierarchical manner. A "master" clock that resides in the suprachiasmatic nuclei (SCN) in the brain synchronizes plentiful clocks present in almost every cell in the body. Specialized neurons in the SCN receive photic information from the retina via synaptic transmission through the retinohypothalamic tract. The oscillators of SCN neurons are extremely robust, as they are tightly coupled through paracrine and synaptic communication of SCN cells; hence SCN oscillators rarely desynchronize in animals deprived of external timing cues and exhibit circadian rhythmicity for months [2]. The SCN then transmits its rhythmic information to cells in various brain regions and peripheral organs via a plethora of outputs. These include neuronal connections, endocrine signals, body temperature rhythms, and indirect cues, provoked by cyclic behavior. For example, rest–activity cycles generate feeding–fasting rhythms, which appear to be dominant *Zeitgebers* for many peripheral organs, such as liver, pancreas, kidney, heart, and skeletal muscles. Although the molecular pathways through which feeding rhythms synchronize peripheral clocks are just emerging, it appears that nutrient-sensing hormones and intracellular metabolites are involved in this process [3].

During the last decade, major efforts have been dedicated to uncover the core clock molecular circuitry (Fig. 1). It is believed that the master and peripheral oscillators share similar clock gene circuitry for rhythms generation, and tick in a self-sustained and cell-autonomous fashion. The rhythms-generating molecular machinery is responsible for ~24 h cycles in gene expression and various physiological and metabolic processes, both in living animals and cultured cells. The core clock circuitry functions based on a negative transcription–translation feedback loop, and is driven by the function of the activators BMAL1 and CLOCK and the repressors PERs and CRYs. BMAL1:CLOCK, heterodimers bind to and activate E-box promoter elements present in the regulatory regions of core clock and clock controlled output genes. The clock genes *Per* and *Cry* are activated in this manner and accumulate in the nucleus forming nuclear complexes that inhibit BMAL1:CLOCK mediated transactivation. PERs and CRYs proteins are subsequently degraded by ubiquitin-dependent pathways, and BMAL1:CLOCK are released to initiate another cycle. The nuclear receptor families ROR and REV-ERB generate a second transcription–translation feedback loop. These proteins are transcriptionally activated by BMAL1:CLOCK, and in turn regulate the activation (ROR) and the repression (REV-ERB) of *Bmal1* and *Clock* expression. In addition, multiple post-transcriptional/translational mechanisms, comprising of mRNA stability, protein translation rate, and protein modifications (e.g. phosphorylation, acetylation) have been implicated in the function of the clock [1,2,4].

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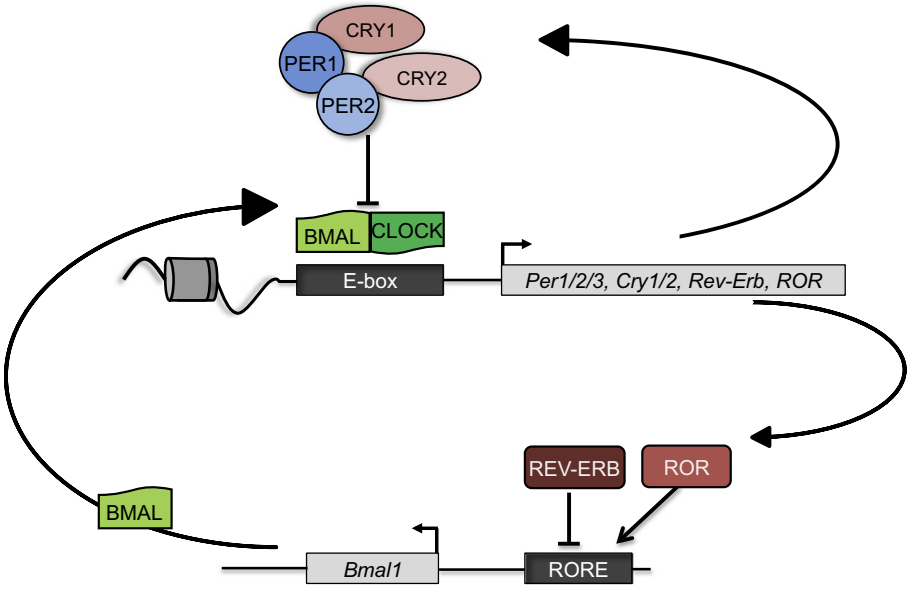

Fig. 1. The molecular core clock circuitry. A schematic depiction of the transcription–translation oscillator, which consists of two principal feedback loops: BMAL1:CLOCK–PER:CRY and BMAL1:CLOCK–REV-ERB/ROR.

Mounting evidence suggests that circadian clocks play a major role in lipid homeostasis and that disruption of circadian rhythmicity is associated with metabolic syndrome and obesity both in animal models and humans. Numerous detailed and comprehensive reviews have been recently published on the circadian orchestration of lipid metabolism and the possible consequences of its disruption for health and disease [5–9]. In this review article, we discuss in detail the current data specifically related to the role of lipids in circadian control. Since the current literature related to molecular aspects of lipids in the master clock in the brain is scant, we mainly cover how lipids cross talk with molecular clocks in peripheral tissues and cultured cells, with the assumption that both the central and peripheral clocks rely on a similar molecular makeup. We also discuss how recent advances in circadian lipidomics have shed new light on our understanding of mechanisms implicated in circadian regulation alongside lipid metabolism.

2. The effect of lipids on circadian rhythmicity

Studies with genetic animal models for obesity, and nutritional studies in mice and humans, suggest that obesity and hyperlipidemia are associated with disruption of circadian rhythmicity, both at the physiological and molecular levels. These findings hint towards the possible involvement of lipids in circadian control.

2.1. Evidence from genetic animal models for obesity

Genetic animal models for obesity are associated with derailed leptin signaling. Leptin is the product of the *ob* gene; it is a circulating blood hormone that regulates adipose tissue mass by modulating food intake and energy expenditure [10]. The *ob*/*ob* mice carry a mutation in the leptin gene, rendering leptin unable to bind to its receptors. Consequently, these mice become severely obese, hyperphagic with hyperlipidemia, hyperglycemia, and hyperinsulinemia. The *ob*/*ob* mice exhibit disturbed sleep pattern and attenuated diurnal and overall locomotor activity. Reports, however, on the effect of the mutation on the daily pattern of food intake are indecisive [11,12]. While oscillations in mRNA expression of clock genes in the SCN are unaffected in *ob*/*ob* mice, daily rhythms of these genes are attenuated in the liver and white adipose tissue [11]. Likewise, experiments done with mouse model of obesity, diabetes and dyslipidemia wherein leptin receptor activity is deficient due to a point mutation in the gene for the leptin receptor (*db*/*db* mice), showed altered circadian gene expression of several core clock genes in the liver. Interestingly, *db*/*db* mice exhibited arrhythmic behavior under constant darkness while circadian gene expression in the brain was retained [13]. Another animal model is the Zucker rat that harbors a mutation in the leptin receptor. These rats are relatively insensitive to leptin signaling, and consequently exhibit a similar obese phenotype like the *ob*/*ob* and *db*/*db* mice. Body temperature, locomotor activity, and feeding rhythms were phase-advanced in obese Zucker rats [14–16]. Another study done with Zucker rats reported differences between lean and obese rats, in respect to their daily mean and circadian amplitude of body temperature and locomotor activity; however no significant difference was observed in the circadian period length under constant light conditions [17]. An additional study with obese Zucker rats showed that transcript levels of some clock genes decreased significantly around their respective peak expression time in the liver, but not in the SCN, white adipose tissue and the heart [18].

Taken together, these findings are inconclusive and the effects might be indirect as these genetic animal models also exhibit derailed glucose metabolism and various other metabolic abnormalities. Nevertheless, these studies point towards a potential link between hyperlipidemia and obesity with circadian rhythmicity.

2.2. Evidence from nutritional studies—high fat diet

Nutritional studies performed in mice fed with high-fat diet that contains ~5-times more fat (40–60% kcal) than regular chow, showed that it leads to a longer period length of circadian rhythmicity under constant conditions, attenuation of diurnal rhythms in feeding and locomotor activity, and diminution of circadian gene expression in peripheral organs (e.g. liver and adipose tissue) [19]. More specifically, studies in mice and rats revealed, for example, that Oleanolic acid can increase *Clock* and *Bmal1* expression [20]. Recently, the Corsi group performed a high-throughput profiling of the liver metabolome and transcriptome, upon high fat diet [21]. They found that high fat diet imposes reprogramming of the circadian clock, including loss of oscillation of a large number of normally oscillating genes; phase advance of an additional subset of oscillating transcripts; and induction of de novo oscillating transcripts. Notably, the rapid influence of the diet on the clock (i.e. 3 days) in this study suggested that the nutritional challenge, and possibly

lipid themselves, and not merely the development of diet-associated complications such as obesity, are sufficient to alter the clock's function.

High-fat diet was also reported to affect the resetting properties of the master clock in the SCN [22]. Compared to control diet, mice fed with the high-fat diet exhibited impaired time adjustment to photic resetting, for example slower rate of re-entrainment of behavioral and body temperature rhythms after 'jet-lag' test. At the molecular level, in mice fed a high-fat diet, photic induction of both c-FOS and P-ERK in the SCN was markedly reduced. Likewise, hypocaloric diet speeded up re-entrainment to shifted light dark conditions [23,24].

2.3. Evidence from human studies

The effects of obesity and high fat diet on circadian rhythmicity have not been yet thoroughly examined in humans. The current conflicting evidence, emerging from few studies done in humans, emphasize the need for additional well-controlled and comprehensive studies. In white adipose biopsies obtained from morbid obese men, the expression of *hPer2* negatively correlated to waist circumference, a marker of central obesity [25]. Adipose tissue explants from morbid obese women that were cultured during 24 h cycle showed rhythms in expression of clock genes. However, since the oscillations were not compared with aged match lean individuals, these experiments do not exclude the possibility that there might be differences in amplitude and/or phase of the oscillations between obese and lean individuals [26].

A recent study examined the methylation status of CpG sites located in several clock genes (e.g. *Clock*, *Bmal1* and *Per2*) in obese individuals and upon weight loss [27]. Comparison of clock gene methylation features revealed a statistically significant difference in the methylation status of different CpG sites in *Clock*, and to a lesser extent in *Bmal1*, in obese compared to normal weight females. The methylation pattern of different CpG sites in *Clock*, *Bmal1*, and *Per2* exhibited significant associations with parameters such as body mass index and adiposity and correlated with the magnitude of the weight lost.

In summary, the above-detailed literature suggests that obesity and high fat diet can disrupt circadian rhythmicity in mammals, though the underlying molecular mechanisms are far from being identified. These observations imply that lipids are directly or/and indirectly implicated in the clock's function. Recent studies have commenced to shed some light on the possible molecular mechanism implicated in the effect of lipids on circadian rhythmicity.

3. Molecular mechanisms that tie lipids to the core clock circuitry

The molecular mechanisms through which obesity/high fat diet and more specifically lipids affect circadian rhythmicity are just emerging. They range from a general effect on feeding habits to more specific effects of certain lipid species on clock components, through the action of nuclear receptors. Such mechanisms are likely to function in concert and are discussed below.

3.1. Altered feeding–fasting cycles

The tight coupling between circadian and metabolic cycles is supported by the impact of feeding–fasting cycles on the phase of clocks in peripheral organs [28]. As expected from nocturnal animals, mice mostly consume food during the night. Upon inverted feeding, namely when food is provided exclusively during the day, the phase of peripheral circadian clocks gradually adapts to the new feeding regimen, reaching an inverted steady state phase after about 2 weeks [29]. Feeding inversion has very little impact on the phase of the master clock in the SCN. Therefore, feeding–fasting cycles appear to be very prominent timing cues for peripheral clocks, even dominating the synchronization cues emitted by the master clock in the SCN. Obviously, under normal conditions the food and SCN dependent timing cues are not in conflict. Hence, the SCN imposes feeding rhythms and thereby employs food-dependent Zeitgebers in addition to other timing cues (e.g. neuronal, blood borne signals) to set the phase of peripheral clocks [3].

The prominent effect of feeding is also evident from studies comparing circadian gene expression in mice fed ad libitum with mice under time restricted feeding regimen. The Panda group systematically dissected the role of feeding pattern and the circadian oscillator in determining hepatic rhythmic gene expression [30]. They found that feeding time had a profound effect on rhythmic gene expression, as the repertoire, phase, and amplitude of rhythmic transcripts in wild-type livers are products of both feeding pattern and the intrinsic circadian clock. Moreover, temporal consolidation of feeding restored 24 h rhythms in gene expression of plentiful transcripts in oscillator-deficient mice (i.e. *Cry1/2* double knockout mice).

The diet composition appears to have an impact on the rhythmic feeding behavior in mice. Upon high fat-feeding, mice exhibited altered feeding–fasting cycles very similar to clock-deficient mice (e.g. *Cry1/2* and *Per1/2* double knockout mice), as they consumed higher percentage of daily food intake during the light phase, concomitantly circadian gene expression was attenuated [19]. Interestingly, when mice were fed with high fat diet exclusively during the night, circadian rhythmicity in gene expression was restored [31]. This has been shown to have wide implication in respect to obesity and metabolic syndrome, as mice under time restricted high fat diet consumed equivalent calories as those with ad libitum access, yet were protected against obesity, hyperinsulinemia, hepatic steatosis, and inflammation. The time restricted feeding regimen improved CREB, mTOR, and AMPK pathway function and oscillations in expression of circadian core clock and output genes [31,32]. Conceivably, the effect was achieved by restoring feeding–fasting cycles, which in turn consolidated circadian gene expression and circadian activation of various metabolic pathways. Hence, high fat diet, and likely obesity disrupt circadian rhythmicity through dampening of feeding–fasting cycles that serve as an extremely potent Zeitgeber for peripheral circadian clocks.

3.2. Nuclear receptors

In recent years emerging evidence suggest that nuclear receptors play important roles in circadian control. The superfamily of nuclear receptors comprises of highly conserved transcription factors, regulating gene expression in response to various stimuli. Commonly, these receptors' ligands are small, hydrophobic molecules, such as steroid hormones, fatty acids, lipophilic vitamin derivatives and dietary metabolites. Hence, they function as metabolic sensors that participate in the regulation of cellular catabolic and anabolic processes, including lipid homeostasis [33,34].

Twenty out of the 49 known nuclear receptor genes in the mouse genome are expressed in the liver in a circadian manner [35]. These include members of the PPAR (PPARα,β/δ,γ) and the ERR families (ERRα,β,γ), both REV-ERB isoforms (REV-ERBα,β), two ROR isoforms (RORα,γ), FXRβ, SHP, a small heterodimerization partner and antagonist of several nuclear receptors, thyroid receptor (TRα), and orphan receptors serving as immediate early transcription factors in signaling pathways. Remarkably, several nuclear receptors have been implicated in the clock's function at multiple levels. REV-ERBs, and RORs are considered core clock components, whereas PPARs have been demonstrated to modulate circadian rhythms. Other nuclear receptors may affect the clock's function by stimulating or repressing pathways producing putative ligands such as lipid compounds. For example, LXR stimulates whereas FXR and SHP repress the expression of Cyp7a1, encoding the rate-limiting enzyme in the conversion of cholesterol to bile acids [36,37]. These receptors are likely to have impact on the intracellular levels of various sterol compounds, which in turn may suppress, for example, the transactivation activities of the core clock transcription factors RORα and RORγ in the liver.

As sensors of nutrient levels, these nuclear receptors connect the external milieu with intracellular processes; therefore they might play

a role in conveying metabolic input into the core clock circuitry. In the following section we will specifically focus on lipid related nuclear receptors that have been implicated in the clock's function (Fig. 2).

3.2.1. RORs

The Retinoic acid receptor-related Orphan Receptors (RORs) are a subfamily of nuclear receptors that are known to bind specific DNA sequences termed ROR-response elements (ROREs) [38]. Once bound to their element within the promoter of a target gene, all three RORs isoforms (i.e. RORα, RORβ, and RORγ) are capable of recruiting co-factors and modulate its expression [39]. RORα and RORγ display high sequence similarity; both are expressed in the liver and participate in regulation of lipid biosynthesis and absorption [40,41].

Accumulating evidence highlight the importance of RORs both in lipid sensing and lipid homeostasis [39]. The involvement of RORs in lipid regulation has been demonstrated in different genetic mouse models. The *staggerer* mice, which harbor a dysfunctional RORα, exhibit significant aberrations in lipid hemostasis—notably, decrease in serum and liver triglycerides, and in total plasma cholesterol, and high density lipoprotein (HDL) [42]. Similarly, RORα and RORγ double knockout mice present significant reduction in blood triglyceride and cholesterol levels [40]. At the molecular level, it was shown that RORs affect the expression of several master lipid regulators such as SREBP1c and multiple genes encoding lipid metabolic enzymes [43,44]. Over a decade ago, crystallography studies of RORα identified cholesterol bound within its ligand-binding domain, suggesting that it is in fact the receptors' natural ligand [45,46]. More recent studies showed that the binding of RORα and RORγ to certain oxysterols, in particular 7-oxygenated sterols [47] and 24S-OHC [48], can regulate their transcriptional activity. These observations reveal a feedback loop in which ROR activity is modulated by various lipids derivatives and in turn RORs have a role in lipid metabolism.

Several lines of evidence suggest that RORs play a critical role in the clock's function. The *staggerer* mice exhibit aberrant locomotor activity and unstable circadian rhythmicity [49], together with altered temporal feeding behavior [50]. Moreover, conserved ROREs are found in several clock genes' promoters, and RORs have been shown to positively support transcription oscillations in the expression of *Bmal1* [51]; *Rev-Erbα* [52] and *Npas2* [53]. Notably, in several peripheral tissues, including the liver, the expression of RORα and RORγ is clock-dependent, and controlled by BMAL1:CLOCK heterodimers and REV-ERB nuclear receptors [35,51]. Concomitantly, the RORβ isoform is primarily expressed in a circadian manner in various regions in the central nervous system, including the SCN. RORβ mutant mice exhibit extended period length in locomotor activity under constant darkness [54,55].

Taken together the above-described studies indicate that RORs are key members of the clock feedback loop alongside their function as receptors for lipids; hence it is conceivable that they might play a role in lipid control of circadian rhythmicity.

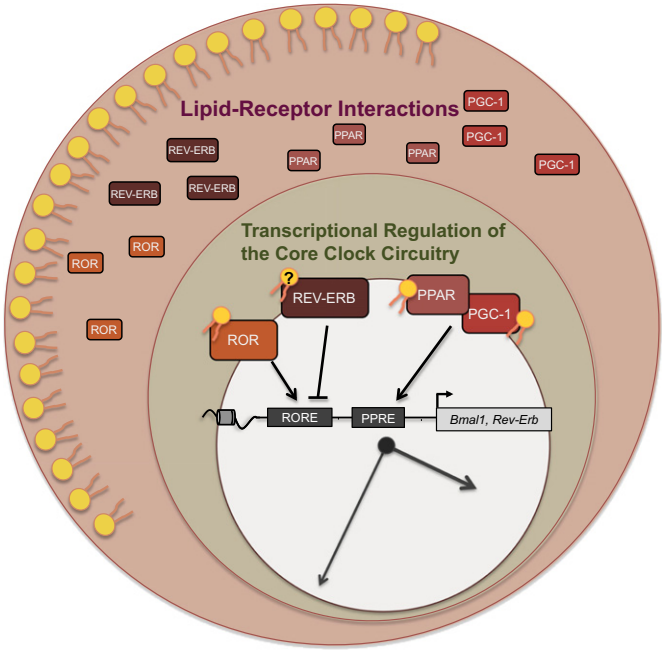

Fig. 2. Nuclear receptors in circadian control—the potential role of lipids. A schematic depiction of molecular mechanisms implicated in regulation of circadian clocks by lipids, primarily through the activation of nuclear receptors and their co activators. As discussed in the relevant sections, these mechanisms likely function in concert, with complex interplay between the different nuclear receptors, which is not emphasized in this figure.

3.2.2. PPARs

The mammalian peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that are expressed in various tissues. The PPAR protein family consists of several isoforms: PPARα, PPARβ/δ, and PPARγ. The isoforms are different in terms of tissue distribution and ligand specificities, and each of them activates or suppresses multiple genes involved in lipid and glucose homeostasis [56]. PPARs also exhibit tissue specificity in respect to their circadian expression. For example, PPARα transcripts cycle in white adipose, brown adipose, and liver but not in muscle. PPARγ selectively cycles in white adipose and liver, whereas PPARδ transcripts oscillate exclusively in brown adipose and liver. Furthermore, there are significant differences in PPAR peak accumulation phases among different tissues [35]. The circadian expression of *PPARα* is regulated through CLOCK:BMAL1 transactivation via E-box-rich region located in the second intron of the *PPARα* gene [57]. Alternately, the transcriptional activity of PPARγ protein was found to be repressed through interaction with PER2, and consequently alter lipid metabolism in *Per2* null mice [58]. Likewise, PER3 was also reported to suppress the transcriptional activity of PPARγ [59].

Similar to RORs, PPARs are not only regulated by circadian clocks—they also appear to play a prominent role in maintaining circadian rhythmicity. Both PPARα and PPARγ were reported to regulate circadian gene expression, through rhythmic binding at upstream regulatory element (PPRE) present in core clock genes. PPARα was shown to regulate *Bmal1* expression both in the SCN and in peripheral organs such as the liver [60]. Interestingly, PPARα binding on *Bmal1* gene regulatory region overlaps with PER2 binding and correlates with activation of *Bmal1* expression [61]. Similarly, PPARγ was demonstrated to directly regulate the circadian expression of *Bmal1* [62] and circadian behavior and metabolism [63]. In addition, both PPARα and PPARγ were found to directly bind response element within the *Rev-Erbα* gene and regulate its expression [64,65].

It is likely that PPARs' role in circadian regulation is influenced through their interaction with fatty acids and sterols, since various fatty acids and eicosanoids are considered as natural ligands for PPAR activity [66–69]. Stimulation by the lipid ligands triggers these receptors' interaction with either their co-activators and/or their binding to DNA regulatory elements. Indeed, Shirai et al. tested the effect of bezafibrate, a PPARα ligand, on circadian locomotor activity and found that in mice bezafibrate can induce changes in the onset of activity and in circadian gene expression, independently of the SCN (i.e. in SCN ablated mice) [70]. Likewise, the effect of high fat diet on circadian gene expression has been proposed to be mediated in part through PPARγ effect on BMAL:CLOCK function [21].

In conclusion, PAPRs are positioned at the junction of lipid metabolism and circadian rhythmicity. These transcription factors are simultaneously controlled by lipids and regulate lipid metabolism; concomitantly they emerge as important players in supporting circadian rhythmicity and are regulated by circadian clocks.

3.2.3. PGC-1α

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is an inducible, broad and potent transcriptional co-activator. PGC-1α was first identified through its functional interaction with PPARγ in brown adipose tissue [71]. Since then, multiple PGC-1α partners have been identified in various tissues [72]. PGC-1α is expressed in tissues with high oxidative capacity, where it regulates cellular energy-related metabolic pathways, including mitochondrial biogenesis and respiration, thermogenesis, and gluconeogenesis [73].

In view of its pivotal role in coupling environmental cues with metabolism, the involvement of PGC-1α in circadian rhythms was examined [74]. PGC-1α is rhythmically expressed both in liver and muscle. Interestingly, overexpression of PGC-1α in primary hepatocytes induced the expression of core clock genes such as *Bmal1*, *Clock*, *Rev-Erbα*, and *Rev-Erbβ*. Moreover, experiments performed with PGC-1α null mice evinced prolonged period length of locomotor activity under constant conditions, and blunted diurnal oscillations in oxygen consumption rate.

PGC-1α expression and activity are highly responsive to the cellular metabolic state. Hepatic PGC-1α expression levels are elevated in multiple mouse models of diabetes and obesity, including leptin-deficient [75], and insulin receptor knockout mice [76]. Furthermore, several studies both in humans and rodents indicated that PGC1-α expression levels are modulated in response to starvation [75,77], high fat diet [78,79], and by direct infusion of lipids, which decreases the expression of PGC-1α in healthy human subjects [80]. Concomitant with these findings, loss of only one allele of hepatic PGC-1α was sufficient to cause significant dysfunction in fasting lipid oxidation, ketogenesis, and misregulation of circulating triglyceride levels. Analysis of hepatic gene expression suggested that fatty acid oxidation and lipid processing pathways were highly affected in the absence of PGC-1α [81].

Taken together, although PGC-1α does not appear to bind directly lipids, its important function in regulating lipid homeostasis and its functional interaction with PPARγ, further lend support to the possibility that PGC-1α might play a role in coupling signals originating from lipid signaling to the core clock circuitry.

3.2.4. REV-ERBs

REV-ERBs (i.e. REV-ERBα and REV-ERBβ) exhibit exceptional similarity in structure and differ from most nuclear receptors as they lack a ligand-activating domain [82]. Both REV-ERBα and REV-ERBβ share an extensive overlap in respect to their genomic targets, including genes that are implicated in lipid metabolism and many other metabolic-related genes [83,84].

REV-ERBα has been identified already a decade ago to participate in the clock's feedback loop as a negative regulator of *Bmal1* expression [85,86]. Its activity was shown to counteract that of the positive regulators of *Bmal1* expression, namely RORs, by competing for genomic binding sites (ROREs). In turn, the expression of *Rev-Erbs* was found to be regulated by CLOCK:BMAL1 in a circadian manner [35]. For a long time, however, its significance was shaded by the mild circadian phenotype of the *Rev-Erbα* null mice. Only upon overcoming the redundancy between the two isoforms their true importance was unraveled. Consecutive studies with REV-ERBs double knockout mice demonstrated their principal role in the core clock circuitry as these mice exhibited arrhythmic gene expression, aberrant locomotor activity and metabolic disorders [83,84,87].

REV-ERB was reported primarily to bind and serve as a heme sensor [88,89]. Yet, in view of its joint function with other lipid-sensitive nuclear receptors, it is tempting to speculate that lipids themselves might also modulate REV-ERB activity indirectly or even directly. In this conjuncture, it is important to note that several lipid-responsive nuclear receptors, earlier described in this section, such as ROR and PPARγ together with PGC-1α were reported to regulate *Rev-Erb* expression and that high fat diet was indeed found to alter its expression [19].

In summary, the aforementioned examples only partially decipher the various and likely redundant and intricate mechanisms through which lipids are integrated into the clock's machinery. They do highlight the complexity whereby lipids can intertwine with the circadian rhythmicity as these nuclear receptors are both responsive to lipid ligands and control lipid homeostasis. Concomitantly they are regulated by circadian clock, and in turn participate in circadian control. Further studies are expected to shed more light on the different mechanisms and dissect their specific contribution to circadian rhythmicity.

4. Circadian clocks in the lipidomics era—old questions new insights

During the last decade, extensive transcriptome profiling performed throughout the day in liver and additional peripheral organs has demonstrated the pervasive circadian control of physiology and metabolism [90]. Circadian transcriptomes are considered as a reliable readout for circadian rhythmicity and have been instrumental in highlighting metabolic pathways that are potentially under circadian control. In recent years, in view of the expansion of advanced technologies, several labs commenced employing high throughput metabolomics and lipidomics approaches to study circadian rhythms. These recent advances have paved new avenues in circadian research and evinced multiple layers of regulation and complexity in circadian control.

4.1. Circadian metabolomics

Metabolomics enable the systematic study of small functional molecules involved in biological processes. This technology jumpstarted the progress from studying individual compounds to exploring a broad combination of well-defined metabolites and even to the identification of novel previously uncharacterized metabolites. Ueda et al. were among the first to introduce metabolomics approaches to the chronobiology field. They quantified the spectra of hundreds of metabolites throughout the day, first in mouse [91] and more recently in human blood samples [92]. They successfully established a reliable metabolite timetable method to determine internal body time using these profiles. Notably, among the oscillatory peaks detected in mouse blood samples, 14 were identified as various species of lysophosphatidylcholines with different unsaturated fatty acids. Lysophospholipids also exhibited circadian oscillations in human blood samples, but reached their zenith levels in the opposite time compared to mice. This likely reflects the differences between mice that are nocturnal animals versus humans that are diurnal. These works have primarily focused on employing blood metabolomics as a signature for internal body time, and to a lesser extent aimed at identifying these metabolites, their metabolic pathways and circadian regulation.

Consecutive studies performed targeted metabolomics, aiming to identify metabolic pathways that exhibit diurnal oscillations and dissecting their circadian control. Metabolomics studies of plasma and saliva samples from humans revealed that 15% of all identified metabolites oscillate in a circadian manner independently of scheduled sleep and food intake [93]. Remarkably, a high proportion of rhythmic metabolites in blood plasma were fatty acids. Likewise, Raynaud et al. applied metabolomics to examine daily oscillations in blood plasma metabolites in human individuals under normal conditions and identified daily oscillation in several lipid species including acylcarnitines and lysophospholipids [94]. Similar studies conducted with mouse liver samples from wild type and clock mutant mice identified clock controlled circadian oscillation of various groups of metabolites, including lipids and more specifically fatty acids [95]. Recently, an integrative database was launched (CircadiOmics database) that integrates circadian genomics, transcriptomics, proteomics and metabolomics [96] facilitating the use of the current available data for deciphering circadian control of various metabolic pathways. It should be noted that in contrast to the high amplitude oscillations in transcripts abundance evinced by numerous transcriptome studies, diurnal changes in metabolite levels

appear to be significantly shallower. For example, metabolite oscillations in human blood samples ranged around 2 fold [93,94] and most lipid species in the liver oscillated with an amplitude of about 1.5 fold [97]. This also applies for proteomics datasets in which for most cases the amplitude of protein oscillations is relatively shallow [98–100]. The striking difference between the high amplitude oscillations in transcript levels versus the shallow oscillations detected for protein and metabolites such lipids reflects the multiple layers of regulation that participate in their accumulation. In fact, metabolites are likely to exhibit even higher complexity than proteins, because their production requires multiple steps that largely depends on the enzymatic activity of a number of proteins, and relies on substrate/co factor availability.

Circadian metabolomics, therefore, do not only serve as a reliable readout for endogenous time, but also appear to be a powerful tool that can be used to study the cross talk between metabolism and circadian clocks. As detailed below circadian lipidomics emerge as a good example for such a case.

4.2. Circadian lipidomics

Lipidomics describes the complete lipid profile within a cell, tissue or organism, and is a subset of the "metabolome" which includes other major classes of biological molecules (e.g. proteins/amino-acids, sugars and nucleic acids). The "Circadian lipidome" is of a particular interest, as aforementioned lipid homeostasis appears to be under circadian control and disruption of circadian rhythmicity is associated with dyslipidemia and obesity [101,102]. Various clock mutant mouse models exhibit altered lipid metabolism. These include elevated VLDL triglyceride levels in *Rev-Erbα* null mice [103], dampening of triglycerides oscillations in blood plasma of *Bmal1* $^{-/-}$ mice [104], hyperlipidemia and hepatic steatosis in *Clock* mutant mice [105], and reduced whole body fat, and total triglycerides and fatty acids in blood plasma of *Per2* $^{-/-}$ mice [58].

Circadian lipidomics analyses are still in their infancy, yet in recent years few studies have been published. A circadian lipidomics analysis was performed on human blood plasma to determine the time-course concentrations for 263 lipids [106] and found that ~13% of lipid species exhibited circadian variation. Most of the lipids that were identified as rhythmic were triglycerides and diglycerides, and peaked around the circadian time (CT) 8 (i.e. 8 h after lights were turned on), namely the subjective lunchtime. This is in agreement with the phase observed for lipid accumulation in a previous human plasma metabolome analysis [93]. Diurnal changes in triglycerides were also observed in blood plasma of rats. Total plasma triglyceride levels were 2-fold higher at midnight (CT 18) [107]. In both human and rodents, oscillating triglycerides in blood plasma reached their peak levels during the active phase (i.e. in human during the light and in rats during the dark phase). We have recently performed a comprehensive circadian lipidomics analysis on mouse liver and identified that ~17% of 159 quantified lipids display circadian rhythmicity [97]. Interestingly, the majority of the oscillating lipid species were triglycerides (~33%) and reached their zenith levels around the subjective light phase (i.e. CT8). The findings that triglycerides accumulate in rodent plasma during the active phase and in liver during the rest phase, may suggest that triglyceride levels build up in different phases in liver and blood in a manner that likely depends on feeding-fasting cycles and circadian clocks. As food is a major source for triglycerides, it is reasonable that they accumulate first in the blood during the active phase, upon food ingestion and subsequently deposited in peripheral organs such as the liver during the rest phase.

4.3. New insights on circadian rhythms from circadian lipidomics

As described above the circadian lipidome can serve as a reliable readout for circadian rhythmicity, similar to transcriptomes. Likewise, it can also provide a profound insight regarding the mechanism participating in lipid homeostasis and their temporal organization. For example, the glycerol 6 phosphate pathway is the principal pathway for triglyceride biosynthesis in the liver. Previous transctipome studies have demonstrated that multiple enzymes within the glycerol 6 phosphate pathway are expressed in a circadian manner, however they exhibited a wide range of expression phases. Thus, solely based on the mRNA expression data it would have been difficult to predict whether the final products (i.e. triglycerides) would accumulate in a circadian manner and identify their actual phase. Our recent lipidomics analysis clearly showed that in contrast to the disparate expression of genes encoding enzymes that are involved in triglyceride homeostasis, all oscillating triglycerides peak around CT8 in the liver of wild type mice [97].

An even more exciting possibility is that circadian lipidomics might shed new light on our understanding of the clock's function and uncover novel mechanisms implicated in circadian rhythmicity. For example, it is possible that by identifying oscillating lipids species, we might be able to unravel novel components that might function as signaling molecules that feedback to clock circuitry, similar to what has been previously shown for NAD^+. Circadian clocks regulate the daily oscillations in NAD^+ levels [108,109], which in turn regulate circadian oscillations, through modulating the DNA binding activity of BMAL1:CLOCK [110] and the NAD^+-dependent enzymatic activity of SIRT1 and PARP-1 that are implicated in the clock's function [29,111–113].

Interestingly, we found that a similar fraction of lipids (~17%) were oscillating in both wild type and *Per1/2* null mice fed ad libitum, most notably triglycerides. However, they largely differed in their accumulation phase and composition [97]. These observations are intriguing as mice lacking both PER1 and PER2 are arrhythmic under constant darkness and their circadian expression of core clock genes is largely abolished. This raises the question; what are the molecular mechanisms that drive the circadian oscillations in triglyceride accumulation in the absence of a functional clock. We do show that feeding-fasting cycles can strongly shape the phase of triglyceride accumulation in mouse liver and that *Per1/2* −/− mice differ in their eating habits compared to wild type mice. However, their feeding behavior cannot fully explain the oscillations of triglyceride in these mice, as they consume equal amount of food throughout the day, and hence food ingestion cannot serve as a timing cue. Hence, our lipidomics analysis raised the possibility that there might be additional mechanisms that retained circadian rhythmicity even in the absence of a functional clock, an observation that so far did not emerge form transcritpome studies. It is evident that further studies are required to identify the molecular mechanisms that drive the circadian accumulation of triglyceride in the absence of a functional clock.

This begs the question whether our current view of circadian clocks has been biased during the years primarily towards the transcription level, and that circadian oscillations in gene expression simply pour down the stream as a funnel to protein abundance and metabolite accumulation. This is similar to the consensus view that prevailed for many years that the mammalian timekeeping function was limited to the SCN that integrates environmental cues and communicates the information to the rest of the body [114]. Although the SCN clearly plays a major role in circadian rhythmicity and synchronizes the time in peripheral organs, it is now recognized that most individual cells within peripheral tissues have self-sustained rhythms in gene expression that persist even in culture [115]. It is therefore tempting to speculate that rhythms in transcript abundances indeed drives rhythms in other cellular molecules (i.e. proteins, metabolites), but that circadian clocks might also developed independently, in parallel, for proteins and different metabolites such as lipids (Fig. 3).

5. Summary and future directions

Remarkable progress has been made in our understanding of the mammalian circadian timing system during the past decade. This is in part thanks to the flare-up in circadian expression datasets that carried a tremendous value. They demonstrate the pervasive circadian control

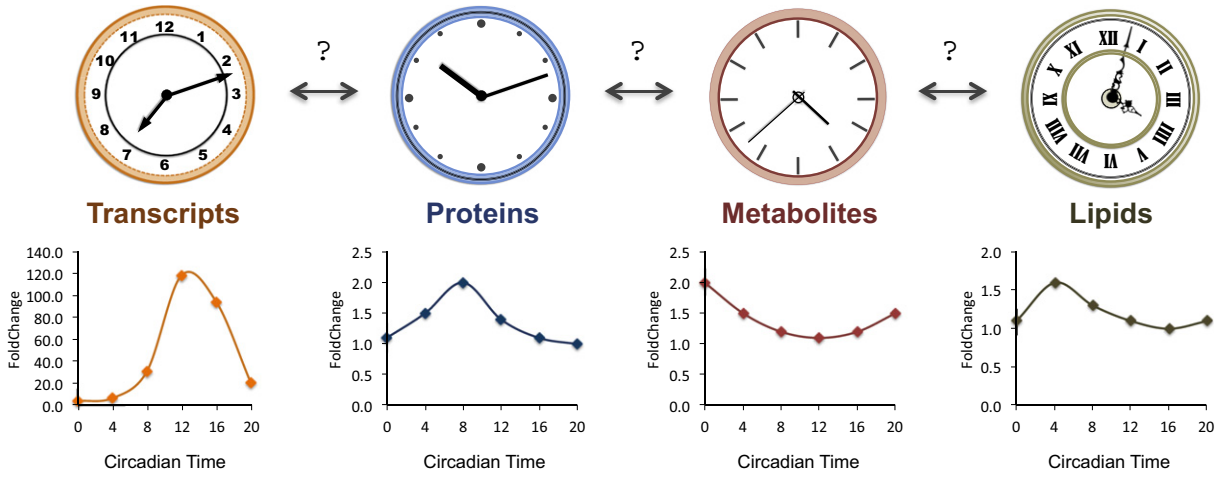

Fig. 3. Circadian information integrates with multiple layers. A simplified model depicting the possible inter-relation between different circadian expression readouts: RNA transcripts, protein, lipids and metabolites levels.

of metabolism and physiology, in addition to consolidating our fundamental understanding of the core clock transcriptional feedback loop. Nevertheless, additional layers of complexity such as plethora of post-translational regulatory mechanisms have been shown to contribute critically to proper clock functions. In addition, evidence point towards a tight coupling of circadian gene expression to metabolic cycles in mammalian cells. Furthermore, the identification of non-transcriptional circadian clocks in mammals as supported by the recent discovery of oxidation–reduction cycles of peroxiredoxin proteins [116], which persist even in the absence of transcription, suggests that additional and different approaches should be taken in the ongoing quest to uncover molecular oscillators and revise and amend the current prevailing paradigm.

The exciting emerging era of proteomics, metabolomics, and more specifically lipidomics, commence to deepen our understanding of how the clock functions, which pathways are under circadian control, and are expected to advance and modify the way we perceive circadian rhythms. Lipidomics approaches have been already used to identify pathways coupling the molecular clock to fatty acid regulation, identify circadian output in the absence of a functional clock, and dissect the interplay between feeding and clocks in circadian control.

Acknowledgments

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