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The Circadian Clock and Liver Function in Health and Disease

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Abstract

All organisms are subjected each day to changes in the light intensity generated by the Earth's rotation around its own axis. To anticipate this geo-physical variability, and to appropriately respond biochemically, most species, including mammals, have evolved an approximate 24-hour endogenous timing mechanism known as the circadian clock (CC). The 'clock' is self-sustained, cell autonomous and present in every cell type. At the core of the clock resides the CC-oscillator, an exquisitely crafted transcriptional-translational feedback system. Remarkably, components of the CC-oscillator not only maintain daily rhythmicity of their own synthesis, but also generate temporal variability in the expression levels of numerous target genes through transcriptional, post-transcriptional and post-translational mechanisms, thus, ensuring proper chronological coordination in the functioning of cells, tissues and organs, including the liver. Indeed, a variety of physiologically critical hepatic functions and cellular processes are CC-controlled. It is not surprising then, that the modern lifestyle (e.g. travel and jet lag, night and rotating shift work), which force 'circadian misalignment' have emerged as major contributors to global health problems including obesity, non-alcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH). Here, we provide an overview of the CC-dependent pathways which play critical roles in mediating several hepatic functions under physiological conditions and, whose deregulations are implicated in chronic liver disease including NASH and alcoholic liver disease (ALD).

Introduction

The word circadian is Latin in origin, and translates to 'about a day', hence, oscillations of ~24 hours are referred as circadian rhythms. These rhythms are generated by the Earth's 24 hours rotation, which, in turn drives the light-dark cycle. This daily change in the light

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intensity leads to overt rest-activity and feeding-fasting cycles, e.g. human beings are diurnal and conduct most of their activities during the day and rest at night. Teleologically, these rhythms have allowed organisms to anticipate changes in the external environment (e.g. the light-dark cycle), and to respond by adjusting CC-driven physiological functions, e.g. metabolism [1–7]. Accordingly, the CC-controlled behavioral synchronization with feeding-fasting cycles generates diurnal variations in metabolic activities, which, in turn, ensures energy homeostasis. Recent investigations have established that, in mammals, the expression of numerous genes in different organs (including the liver) display circadian rhythmicity, which enables regulation of both anabolism and catabolism [1–6]. Indeed, hepatocyte activities such as nutrient uptake, processing, assimilation and detoxification exhibit remarkable diurnal variations, which enable their alignment with food availability and energetic demand. Physiologically these 'metabolic rhythms' are generated and maintained by the dynamic interactions between the CC and timing cues e.g. light and food (time of eating and its quality). However, our modern lifestyle (jet lag, shift-work, energy-dense foods etc.), which often 'misaligns' CC functioning has recently emerged as prominent contributors to different metabolic diseases and carcinogenesis [8–13].

Here, we focus on how the CC regulates hepatic metabolism to maintain homeostasis and, also provide an overview of how deregulation of CC-controlled pathways could lead to the development of non-alcoholic fatty liver (NAFL) and its progression to NASH. Furthermore, we also discuss evidences linking the CC and alcohol-induced liver disease (ALD).

The anatomic and molecular organization of the mammalian CC-system

Retinal photoreceptors (rods and cones) transform photic energy to electrical impulses and convey them to the brain through retinal ganglion cells (RGC). A subset of RGCs expressing the photopigment melanopsin are intrinsically sensitive to the visible spectrum and directly relay the photic signal to a hypothalamic region called the suprachiasmatic nucleus (SCN) [7–8]. Hence, anatomically, the mammalian circadian system is hierarchical, whereby the light-entrained SCN is the 'central' CC. In turn, the SCN-CC by utilizing humoral and neuronal mechanisms communicates the 'time cue' (a. k. a; 'Zeitgeber'; ZT) to other organs, thereby enabling the synchronization of peripheral CCs (PCCs) [1–8].

At the molecular level, the components of the central SCN and PCCs are the same and are identically organized in multiple transcriptional-translational feedback systems (Figure 1) and generate a cell autonomous self-sustained CC-oscillator with a periodicity of ~24 hours [1–6]. The heart of this oscillator is constituted by a heterodimer of transcription factors (TFs), the Brain and Muscle ARNTL-Like protein1 (BMAL1) associated with the Circadian Locomotor Output Cycles Kaput (CLOCK), which activate genes containing E-Box DNA Binding Sequences (DBS) in their promoter-enhancer regions, including those of *Period* (*Per1*, *Per2*) and *Cryptochrome* (*Cry1, Cry2*) genes. In turn, PER1/2 and CRY1/2 proteins heterodimerize to inhibit the transcriptional activity of the BMAL1/CLOCK-complex (the first-loop of the oscillator), thereby eventually suppressing their own expression (Figure 1). BMAL1/CLOCK-also binds to the E-Box DBSs present in the genes of the nuclear receptors *Rev-Erbα* and *Rev-Erbβ* to activate their transcription, while the presence of RORE DBSs in the *Rev-Erbα/β* genes mediate their autorepression. REV-ERBs also

inhibit (through RORE DBSs) the transcription of their activators *Bmal1* and *Clock,* thus constituting the second-loop of the CC-oscillator. At the beginning of the active phase, levels of REV-ERBs (which are repressors of transcription) decrease, while simultaneously protein levels of transcriptional activators RORα/γ increase, which then bind to RORE DBSs present in *Bmal1* and *Clock* and activate their transcription, thereby initiating the next round of CC oscillation. Additionally, BMAL1/CLOCK-induces expression of the transactivator D-Box binding protein (*Dbp*). The DBP activator and the E4BP4 repressor (which is activated by RORα/γ and repressed by REV-ERBα) competes for binding to D-Box DBSs present in several CC-controlled genes (CCGs).These inter-connected feed-back loops generate circadian oscillations of the expression of ~20% of the genome, such that CCGs containing RORE DBS are transcribed during the active phase, while E-Box and D-Box DBS-bearing genes are expressed during the rest phase[1–6,14]. Moreover, post-translational modifications of CC-components also aids in further fine-tuning of the CC-oscillator functioning. Thus, utilizing multiple mechanisms the CC-oscillator drives a temporally-restricted gene expression pattern, which lies at the core of generating distinct biochemical outputs in individual organs.

Feeding cycles and peripheral clocks

Establishment of feeding cycles as the prominent *zeitgeber* for peripheral tissues, including the liver [15–16], has revealed the existence of extensive cross-talk between metabolism and the CC, and the list of mechanisms through which metabolic signals influence CC functioning and, *vice versa*, are increasing rapidly [1–5]. The dominance of feeding cycles on the liver-clock was demonstrated in 'arrhythmic' *Cry1/2* mutant mice, in which an imposed feeding regime recovered 'rhythmicity' in circadian gene expression pattern [17]. Furthermore, changing the feeding time from active to rest phases in mice shifts the PCCs by ~12 hours [15–16], driven by metabolic alterations acting through PPARα and CREB [18]. Notably, high-fat diet (HFD)-induced 'reprogramming' of the hepatic CC [19] can be prevented by restricting HFD feeding during the active phase [20].

One physiological example of CC-metabolism crosstalk is provided by BMAL1/CLOCK-dependent transcription of the nicotinamide phosphoribosyl transferase (*NAMPT*) gene, which is involved in NAD^+ synthesis [21–22]. CC-dictated NAMPT expression ensures not only a rhythmicity in NAD^+ synthesis but also regulates the activities of NAD^+ -dependent proteins [1–5], e.g. the SIRT1 deacetylase and the ADP-ribosyltransferase PARP-1. In turn, SIRT1 determines: (i) the activity of BMAL1/CLOCK-complex towards their target genes and, (ii) the stability of PER2 protein, which together maintain CC-oscillator functioning [1–5]. Akin to NAD^+, feedback regulation between the CC and heme biosynthesis has also been demonstrated [23–24]. Thus, by controlling metabolite sensors (NAD^+, heme etc.), the CC gauges the cellular energetic and redox status to reset the oscillator with metabolism. Altogether, these investigations have established metabolism as a critical modulator of PCCs.

Circadian regulation of hepatic functions

Given the centrality of the liver in maintaining whole-body physiology, several high-throughput circadian time-course studies have been performed in mouse models investigating cistrome [25–28], transcriptome [29–30], proteome [31–33], and lipidome [34–35].

Circadian transcriptome analyses have revealed two broad crests of transcription in liver, corresponding to the transition between successive active and rest phases [1–5]. Cistromic analyses [25–28] revealed that these two distinct mRNA repertoires are generated due to the rhythmicity of the CC-oscillator, which enables periodic recruitment/removal of TFs and coregulators to epigenetically alter the chromatin landscape of CCGs. Cellular processes like DNA repair, ribosome biogenesis, autophagy, ER-stress are also subjected to circadian regulation, but mainly at the post-translational level [31–33]. Altogether, these investigations have revealed an unprecedented level of CC-control on hepatic physiology (Figure 2). Importantly. deregulation of these CC-regulated pathways/processes has been shown to contribute to the development of NAFLD and other diseases.

The circadian clock and pathophysiology of NAFLD and NASH

Over the last decades, life-style changes have shifted health care priorities world-wide from infectious to metabolic diseases [36–38]. In the context of liver disease, vaccination can now prevent hepatitis B virus (HBV) infection and antivirals can control chronic HBV infection [39–40,43], and recently developed direct-acting antivirals can cure chronic hepatitis C virus in a large majority of infected patients [41–43]. In contrast, the prevalence of metabolic liver diseases such as NAFL and NASH are increasing dramatically in conjunction with obesity and type II diabetes [36–38,44]. NAFLD, is a continuous spectrum of disease initiated by excessive triglyceride (TG) accumulation in the liver. In the absence of concomitant inflammation and hepatocytic injury, this state is largely benign and commonly referred as nonalcoholic fatty liver (NAFL) or simple steatosis [36–38, 45]. However, chronic NAFL usually drives simple steatosis to steatohepatitis (NASH), which is typified by concomitant presence of both lobular inflammation and hepatocellular damage (ballooning). Moreover, NASH predisposes to fibrosis, progressing to cirrhosis and hepatocellular carcinoma (HCC) [36–38]. Like every other aspect of the metabolic syndrome, development of NAFLD and NASH is highly complex which has been reviewed extensively elsewhere [36–38,46–48]. Almost two decades earlier the ‘two-hit’ theory [49] was posited to explain NASH pathogenesis. This theory proclaimed that unrestrained TG deposition in the liver (first-hit; NAFL) leads to ‘secondary hits’ such as oxidative stress, which ultimately leads to NASH. However, with increasing knowledge of metabolism and associated pathologies, NAFLD is now considered as a multi-factorial systemic metabolic disorder [36–38]. Indeed, investigations have revealed crucial roles for intestine, adipose tissue and muscle in NAFLD development. Importantly, insulin resistance also plays a critical, if not indispensable role in NAFLD [36,38,45].

Systemic energy homeostasis is maintained by communications between numerous intra- and inter-organ signaling networks and at the core of NAFLD pathogenesis lies the inability of the liver to effectively metabolize carbohydrates and fatty acids [36–38, 50]. The pathology of NAFLD is generally initiated by perturbations in free fatty acid (FFA) metabolism, which drives excessive TG accumulation in hepatocytes [36–37]. Increased FFA release from adipocytes due to insulin resistance [51] and conversion of excess carbohydrates to FFA via hepatic *de novo* lipogenesis (DNL) [52] are two major sources of TG deposition during NAFLD development, in addition to excess caloric intake. In hepatocytes, FFA can either undergo β-oxidation or be re-esterified as TG. In turn, this pool of TG can either be exported

as VLDL particles or stored in lipid droplets [36–38]. The capacity to metabolize FFA through either β-oxidation or TG formation when overwhelmed (perturbation of dynamic lipid fluxes), leads to the accumulation of lipotoxic species. This buildup of lipotoxic molecules in turn damages hepatocytes through several pathways; e.g. enhanced ERand oxidative-stress, a dysfunctional unfolded protein response (UPR), and inflammosome activation to finally lead to NAFLD development [37,50,53–54]. In the subsequent sections we describe some of these hepatic functions and processes which show diurnal variations and whose deregulation could predispose towards NAFLD/NASH (Figure 3).

Circadian control of glucose metabolism

The liver is the principal gluconeogenic organ in mammals, and participates, along with several other organs, to maintain homeostatic blood glucose levels. The CC sustains the physiological levels of blood glucose by synchronizing tissue-specific mechanisms of glucose metabolism. Accordingly, the SCN-clock controls the feeding/fasting rhythms, while PCCs (liver, pancreatic β-cells, skeletal muscles) drive temporally coordinated gene expression programs to maintain physiological levels of glucose in blood [55].

One of the first studies indicating a role for the liver-CC-oscillator in glucose metabolism showed that *Bmal1* ablation in hepatocytes reduced expression of the glucose transporter (*Glut2*), leading to a decreased post-absorptive glucose uptake in mutant mice [56]. Post-hepatocytic entry, glucose is phosphorylated to glucose-6-phosphate (G6P), which can be either used (through glycolysis or hexose monophosphate pathway) or stored (glycogen synthesis). Remarkably, the CC influences all these processes [2,6]. For example, the hepatic expression of glucokinase (Gck), which controls both glycolysis and glycogen synthesis is rhythmic reaching its zenith during the transition from the rest-phase to the active-phase [17, 57] and temporally matches the surge of postprandial insulin secretion from the pancreas. This increase in insulin secretion also leads to a pulsatile glycogen synthase kinase 3 (GSK3) activity in liver [17], which, in turn, determines: (i) the enzymatic activity of glycogen synthase, (ii) the activity of the glycosylating enzyme O-linked N-acetylglucosamine transferase (OGT), thereby, generating rhythmicity in the glycosylation levels of numerous proteins [58] and, (iii) the stability of REV-ERBα [2,4], which in turn dictates the expression of many CCGs. By controlling the expression of trans-activators *Klf10* [59] and *Hnf4α* [60], the liver-CC further dictates transcription of several genes, which are involved in glucose metabolism.

The CC also controls glucagon-induced gluconeogenesis in liver by regulating the duration of hepatic cAMP production [61]. It was demonstrated that the interaction of the CC-component CRY1 with the regulatory α-subunit of the glucagon receptor blocks hepatic cAMP accumulation during the circadian active phase, thus leading to a temporally-restricted (between rest- and active-phases) activation of the gluconeogenic transcription factor CREB [55,61]. Moreover, BMAL1-regulates the expression of the *Pgc1α* gene [62], which is a coactivator of the gluconeogenic transcription program [2–4]. Thus, by employing multiple strategies the CC controls diverse mechanisms which co-operate to maintain physiological glucose levels [1–5, 55].

Circadian regulation of liver lipid metabolism

In a seminal study, Turek et al. [63] demonstrated that *Clock* mutant mice are obese and have increased blood levels of cholesterol and TG. Since then, multiple genetic studies in mice models have established the CC as a critical regulator of lipid metabolism [64–66]. Indeed, plasma levels of FFA, TG and cholesterol display diurnal variations, and are altered upon mutations of CC-components. Notably, the liver plays a crucial role in generating these variations in blood levels. Indeed, hepatocyte-specific ablation of *Rev-Erbα/β* was found to increase plasma levels of FFA, TG and cholesterol [27,66]. In this regard, a lipidomic study revealed that TG, phosphatidyl inositol and phosphatidyl choline preferentially accumulate in mouse liver during the rest phase [35]. Mechanistically, the CC controls enzymes that are critically involved in regulating various steps of lipid metabolism. As example, expression of the enzyme ATP citrate lyase, which drives mitochondrial export of acetyl Coenzyme A (acetyl CoA), is maximal at the beginning of the active phase [17]. Cytosolic acetyl CoA is carboxylated by acetyl CoA carboxylase (ACC1) to generate malonyl CoA an essential step in fatty acid synthesis. It is well known that AMPK inactivates ACC by phosphorylation [2–4], and CCs by controlling AMPK 'temporally gate' ACC activity [67]. Furthermore, the liver CC by controlling the transcription of *Elovl3*, *Elovl6*, *Fas* etc. 'times' fatty acid synthesis [2–6]. Moreover, the expression of enzymes regulating β-oxidation (*Cpt1/2*) and ketone-body production (*Hmgcs2*) [68–69], as well as their transcriptional regulators PPAR α and δ are also circadian in nature [60].

Hepatic TG synthesis from glycerol-3-phosphate is a multistep process and expression of several genes (*Gpat2*, *Agpat1/2*, *Lipin1/2* and *Dgat2*) that regulate successive steps of TG synthesis is circadian in nature [35]. Importantly, by controlling the transcription of *Pnpla3* the CC also regulates lipid droplet dynamics [35]. Altogether, in *ad libitum* fed mice livers, a prominent crest and trough of TG levels are observed during the rest (~ZT8) and active phases (~ZT2O). Additionally, REV-ERBα-controlled expression of *Insig2* regulates the activity of SREBP1c, thereby leading to CC-command over lipogenesis [70].

Clock and metabolism of bile acids

Intestinal absorption of lipids requires bile acids (BA), which are synthesized in hepatocytes. Besides lipid absorption, recent evidences have established BA as signaling molecules [71–72]. BA are physiological ligands for FXR and the G-protein coupled receptor TGR5 and can activate signaling modules such as the MAPK-pathway [71–72]. By regulating these diverse signaling networks, BA not only control their own levels but also those of TG, cholesterol and glucose homeostasis [71–72]. BA synthesis is controlled by a transcriptional feed-back loop consisting of the nuclear receptors FXR and SHP and hormone FGF15 (FGF19 in humans) [38,73]. Hepatic expressions of both FXR and SHP [60] and the intestinal secretion of FGF15 are 'clock'-gated [74], which, together, drive the circadian transcription of cholesterol 7α-hydroxylase (*Cyp7a1*), the rate-limiting enzyme in the classical BA synthesis pathway. Moreover, the CC-output regulator DBP controls *Cyp7a1* transcription to restrict its temporal expression [75]. Additionally, by regulating the transcription of both E4BP4 and SHP, REV-ERBα directly regulates the expression of *Cyp7a1*. [76] Altogether, these mechanisms cooperatively generate diurnal rhythmicity in BA levels (Figure 3), which is also observed in humans [77].

Clock-controlled cellular processes and NAFLD

Along with controlling systemic metabolism [2–5], several investigations have indicated a critical role for the CC-machinery in regulating autophagy, ER stress and oxidative stress [78–80], all of which may participate in NAFLD and in its transition to NASH [36–37]. For example, in murine livers, expression of key genes controlling different steps of the autophagic process display circadian rhythms, thereby leading to an overall diurnal rhythm in autophagic activity [78–79]. Consistently, hepatocytic mutation of *Bmal1* impairs the entire autophagic process in murine liver. The CC also modulates the ER stress-induced activation of the UPR-driven gene expression program [78,80]. Physiologically, the UPR is necessary to restore cellular secretory capacity following an accumulation of misfolded proteins in the ER and functions by degrading unfolded proteins and activating the expression of chaperones which enable protein folding [81]. The CC by generating ultradian (lesser than a day) rhythms in the expression of UPR master regulators i.e. the inositol-requiring enzyme1α (IRE1α) and the X-box binding protein1 (XBP1) controls the expression of several genes within the UPR pathway [78,80]. Moreover, by regulating activation of the transcription factor CREBH [82] and expression of the deadenylase CPEB4 [83], the CC extends control over the ER-stress response pathway. Deregulation in reactive oxygen species (ROS)production and scavenging have been implicated in the development of NAFLD and NASH. To avoid the dangers of excessive ROS levels, cells are dependent on anti-oxidant enzymes. Interestingly, expressions and activities of several enzymes, e.g. glutathione reductase, superoxide dismutase, glutathione peroxidase and peroxiredoxins display CC-controlled diurnal rhythms [84–85]. Consistently, in peripheral tissues levels of ROS as well as peroxidized lipids/proteins vary per the light-dark cycle [86]. Thus, it is evident that the ‘clock’ plays a remarkable role in regulating several cellular processes where deregulation have been strongly implicated in chronic liver diseases (Figure 3).

Circadian clock, nuclear receptors (NRs) and NAFLD

The nuclear receptor (NR) superfamily which comprises 48 members in humans, control diverse aspects of physiology including metabolism [87–89]. NRs are transcription factors, which upon ligand (natural and synthetic) binding drive gene expression programs, amongst whom are pathways controlling metabolism. Investigation of the circadian expression patterns of all NRs in four mice tissues, including, the liver [60], revealed that at least 20 of the 41 transcribed NR in the murine liver are expressed in a circadian manner thereby providing a possible molecular link between the clock, NRs, and metabolism. In the liver, NRs control a broad range of crucial hepatic functions and are prominently implicated in NAFLD development [38,88–89]. Here, we briefly discuss a few of these NRs that are not only known to be regulated by the ‘clock’ but also have emerged as therapeutic targets for NAFLD (see below). PPARα regulates β-oxidation and ketogenesis [90] and plays a prominent role in inflammation by trans-repressing NF-κB and AP-1 pathways [91]. Importantly, genetic studies in mice indicate that through this trans-repressive activity PPARα can prevent fibrosis development which is a crucial event in NASH pathogenesis [91]. Ligand activation of PPARβ/δ (which plays a prominent role in lipid catabolism) also prevents hepatic fibrogenesis [92]. The Liver X receptors (LXRs) are transcriptional regulators of cholesterol metabolism and hepatic lipogenesis, and LXR activation lowers atherosclerosis by enhancing reverse cholesterol transport [89]. Whereas LXR could be a

possible antifibrotic target [93], LXR-activation enhances lipogenesis due to LXR-induced activation of SREBP1c activity [93]. Lastly, with the establishment of pleiotropic roles of BAs in metabolic regulation, FXR has gained considerable attention as a therapeutic target for NAFLD (see below). Indeed, hepatic FXR activation reduces lipogenesis and improves fibrosis [72].

Clock, gut microbiota and NAFLD

Besides liver-restricted functions and processes, extrahepatic tissues also play a crucial role in NAFLD. Obesity-associated alterations in the gut microbiota (i.e., dysbiosis) composition and their interactions with the host (intestinal epithelial cells; IEC) have been implicated as an etiological agent in the pathogenesis of metabolic diseases, including NAFLD [94–97]. A mechanism through which changes in gut microbiota composition may promote NAFLD is through increased LPS production and delivery to the liver via the portal circulation [98–99]. In turn, microbiota-derived LPS can perturb hepatic lipid metabolism by modulating the production of short-chain fatty acids and altering the BA pool composition [99], which may influence intestinal and hepatic FXR activity, thus affecting both glucose and lipid homeostasis [99]. Remarkably, the CC by regulating the expression of microbial pattern recognition receptors (e.g. TLRs, NOD2) provides a ‘temporal window’ during which microbiota-signals regulate gene expression to maintain homeostasis [100]. Interestingly, the gut microbiota also displays ‘clock’-controlled diurnal rhythmicity [101–102]. Consistently, circadian perturbations (mutation of CC-components or jet lag) lead to dysbiosis and development of metabolic pathologies [101]. Furthermore, mutation of innate immune genes (*Tlr5*, *Nlrp6*, *Nlrp3*) which play pivotal roles in sensing gut microbiota, modulate metabolic pathologies, including NAFL[95].

Chronopharmacology: detoxification, pharmacokinetics, and dynamics

Considering its overall influence on physiology, it is hardly surprising that clinically relevant pharmacological aspects, e.g. pharmacokinetics (PK) and pharmacodynamics (PD), of many drugs are also governed by the ‘clock’, thereby introducing circadian variations in drug metabolism/detoxification and efficacy [84,103]. One of the most prominent examples of circadian control over pharmacology emerges from its ability to regulate almost every step of xenobiotic detoxification in the liver, including absorption, biotransformation and elimination [84,103–104]. Notably, in humans, hepatic absorption of lipophilic drugs occurs more swiftly in the morning than in the evening [103]. Consistently, expression of several transport proteins which mediate xenobiotic uptake e.g. cationic and anionic transporters (*Oct-1*, *Oatp1*, *Oatp1a4* etc.) display circadian rhythmicity [103]. Classically, xenobiotic metabolism is grouped into three (I, II, and III) phases. Phase I involves biochemical modification of substrates by the CYP450 superfamily of enzymes. Importantly, transcript levels of several members (*Cyp2a4*, *Cyp2a5*, *Cyp2b10*, *Cyp2e1*, *Cyp3a11* etc.) of this family are rhythmic, attaining in mouse liver their peak during the rest phase [84,104]. In phase II, xenobiotics are rendered hydrophilic by conjugation to various small molecules.

Notably, phase II controlling genes (*Sult1c1*, *Sult1d1*, *Gsta1*, *Gsta2* etc.) are also expressed in a circadian manner. The excretion of xenobiotics (phase III) is controlled by different transporter proteins, and several of them (*Mrp2*, *Mdr2*, *Abcg2*, *Abcc2* etc.)

oscillate rhythmically in the mouse liver. This pervasive circadian control of all these phases is molecularly achieved by hepatic CC-driven regulation of TFs, enzymes and transport proteins participating in the detoxification process [84,104]. Hepatic expression of TFs (PXR, CAR and AhR), which bind and metabolize xenobiotics, is rhythmic [60]. Moreover, CC-components (RORα/γ) and CC-output regulators (DBP, HLF and TEF) also transcriptionally regulate the detoxification process [105–106]. Accordingly, mice with ablation of the *Dbp*, *Hlf* and *Tef* genes exhibit widespread deficiencies in both basal and inducible detoxification processes [106].

Circadian control of 'pharmacology' extends beyond the liver and has been reviewed elsewhere [84,107]. 'Timing' is of crucial but less-appreciated factor in drug efficacy. Indeed, 56 of the top 100 best-selling drugs in the USA target the product of a circadian gene [108]. However, most of them are yet to be associated and dosed as per the circadian rhythm. Importantly, clock perturbations resulting from HFD feeding can be rescued by 'properly-timed' pharmacological interventions [109]. Taken together, recent studies (although many in rodent models) suggest that it will be highly prudent to investigate the mechanistic basis of circadian variations in PK/PD, in order to include a 'circadian' component for better therapeutic outcomes [84,107].

Therapeutic impact: CC and pharmacological targeting of NAFLD

While licensed pharmacological therapies are not yet available[36–38], a larger number of approaches and compounds are in preclinical and clinical development. Most therapeutic strategies aim to decrease inflammation, fibrosis, and metabolic substrate availability or to increase their disposal from the liver. Weight loss management or bariatric surgery not only improves NASH, but can also induce fibrosis regression [38, 110]. Considering the key impact of CC-control in regulation of metabolism, it is likely that the molecular targets of several drug candidates are CC-regulated. For example, obeticholic acid (INT-747) which activates FXR, reverses histological features of NASH [111] and the CC is well known to control BA metabolism. Interestingly, FGF19 which is rhythmically secreted from intestine (post-feeding) has efficacy in murine models of NASH [112]. Importantly, treatment with the FGF19 analog NGM282 reduces hepatic fat content in NASH patients [113]. The CC-regulated NRs PPAR-α/β are activated by elafibranor (currently in phase 3 trial), which enhances lipid metabolism, insulin sensitivity and reduces inflammation [114]. Furthermore, FGF21, a direct transcriptional target of PPARα reduces steatosis [37]. Significantly, some other potential NASH-modulating compounds [37–38], e.g. resveratrol (SIRT1-agonist), inhibitors of acetyl-CoA carboxylase (ACC) and FAS, further strengthen the CC-connection to therapeutics.

The intimate relationship between metabolism and the CC, as well as the amenability of the CC-oscillator to a variety of 'resetting' signals [1–7], has spurred investigations to explore the potential of 'clock' modulating small molecules as a possible treatment for metabolic disorders [115–118]. Using high-throughput phenotypic screening or medicinal chemistry approaches several molecules affecting the affect circadian period, phase and/or amplitude have been identified [115–118]. Consistent with the molecular-genetic studies revealing a regulatory role for PERs and CRYs in CC-functioning, several compounds have been found

to affect their levels and alter (mostly lengthening) circadian periods [115–117]. One such compound, KL001 was found to bind CRY proteins which prevented their ubiquitination and proteasomal degradation [116,119]. Consistent with the known role of CRYs in suppressing gluconeogenesis, KL001 administration was shown to improve glucose tolerance in diet-induced obese (DIO) mice [116,120]. Recent investigations have also identified several modulators of CC-components RORα/γ and REV-ERBα/β with therapeutic potential in animal models of metabolic disorders [116–118]. In this regard, agonists of REV-ERBs; e.g., SR9009 and SR9011 [121], and an inverse ROR agonist of (SR1555) [122] were found to improve several metabolic parameters in DIO mice. Amongst identified ROR modulators nobiletin (NOB) was demonstrated to enhance the amplitude of the CC, reduce weight gain, improve energy homeostasis and metabolic parameters in both DIO and genetically diabetic (*db/db*) mice [116–118, 123]. Taken together, these small molecule modulators of CC-components provide an opportunity to further reveal regulatory networks in circadian functioning which could be targeted, alone or in combination, to treat metabolic liver disease.

CC and alcohol-induced liver disease (ALD)

Like NAFLD, pathogenesis of alcohol-induced liver disease (ALD) is complex and arises from interactions between metabolic, environmental and genetic risk factors in heavy alcohol consumers. In the context of alcohol-related disease, the CC has largely been investigated from a neurobehavioral perspective, noting CC disruption in alcohol use disorders and addiction [124–125]. For example, rotating shift-workers have increased alcohol intake and tendencies to engage in binge drinking [126]. Genetic variants in some clock genes are also associated with alcohol dependence and increased drinking in humans [127–128]. Finally, transcript levels of CC genes are significantly lower in peripheral blood mononuclear cells from alcohol-dependent patients compared with healthy control subjects [129]. Together, these studies suggest that CC alterations could promote alcoholic disorders and excessive alcohol consumption.

Investigations using Per2::Luciferase knock-in mice demonstrated that alcohol consumption misaligns peripheral clocks from the master SCN clock [130–131]. Additionally, in liver, chronic alcohol consumption disrupts rhythmic oscillations of several CC components and CC-controlled output genes involved in regulating glucose, glycogen, cholesterol, BA and FFA metabolism [130, 132]. For example, chronic alcohol intake in mice alters the diurnal rhythm in hepatic glycogen content due to dampened and/or shifted oscillations in glucose and glycogen metabolism genes [57, 132] Moreover, liver-specific BMAL1 deletion and chronic alcohol abolish day-night differences in hepatic glycogen content [132]. Alcohol consumption also disrupts rhythmic oscillations in the cofactor NAD^+ [130] required for numerous metabolic functions in the liver, including pathways regulated by SIRT1 and PARP-1. Furthermore, CC disruption (mutation or disrupting the lightdark cycle) enhances alcohol-induced tissue injury in mice. For example, alcohol-induced steatosis is higher in livers of Clock-mutant mice as compared to wild-type mice [133]. Liver-specific deletion of BMAL1 also increases hepatic steatosis in mice treated with chronic plus binge alcohol [134]. Moreover, CC-disruption through weekly 12-h shifts in the light-dark cycle, increases gut leakiness and liver injury in alcohol-fed mice [135]. Importantly, whole

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gut and colon permeability is enhanced in night-shift but not in day-shift workers who consumed moderate amounts of alcohol (0.5 g/kg/day) for only one week [136]. Collectively, these studies suggest that CC disruption may increase risk for liver disease in alcohol consumers. Importantly, as key mechanisms and linkages among alcohol-mediated CC disruption, metabolic dysregulation, and tissue injury emerge, it may become possible to pharmacologically target the CC in ALD patients.

Conclusion and future perspectives

The pathogenesis of NAFL and its progression to NASH, the most-prevalent noninfectious liver disease, is complex and multifactorial. Both genetic factors as well as the environment have been shown to play important functional roles. Perturbations in FFA metabolism, which lies at the core of NAFLD could potentially arise from deregulation of several distinct mechanisms. Remarkably, under physiological conditions most of these processes are governed by the CC-machinery. Consistently, in mice models, either mutation of CC-components [1–5] or change in the feeding time [137] are closely associated with a range of metabolic diseases including NAFL. Importantly, recent investigations have categorically established that in humans ‘circadian misalignment’ has adverse metabolic and cardiovascular consequences [138–139]. Furthermore, epidemiologically, single nucleotide polymorphisms (SNPs) in the *Clock* gene [140] and in several CC-controlled transcriptional regulators (e.g. *Pparγ*, *Stat3*, *Ppargc1α*) [88] and the enzyme *Pnpla3* [141] are associated with the development of obesity, metabolic syndrome, NAFLD and NASH. Most significantly, our *nouveau* life-style (nutrient-dense foods, timing of eating and activity) which continuously interferes with endogenous circadian rhythms is also epidemiologically correlated with increasing incidences of all the hallmarks of metabolic syndrome including, NAFLD [8–12]. Given the socio-economic realities in modern societies, it is difficult to avoid circadian disruption. Thus, in addition to life-style modification, CC-targeting approaches may provide therapeutic opportunities overcoming these challenges. Furthermore, comprehensive systems level investigations of the circadian system elucidating physical- and genetic-interaction networks will reveal novel targets to prevent and treat chronic liver disease.

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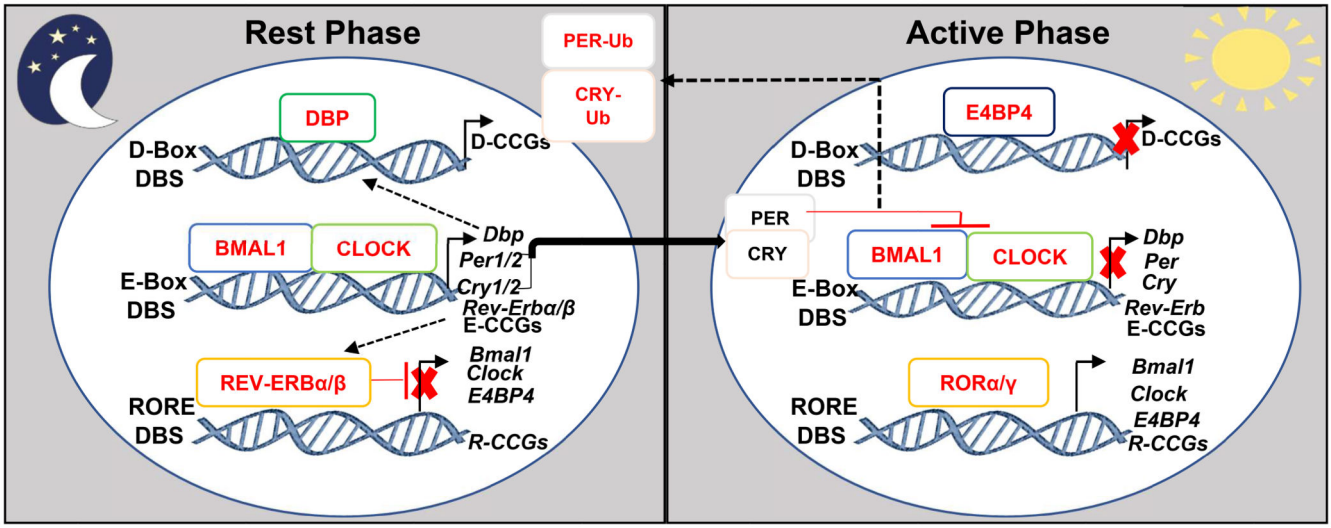

Figure 1. The molecular architecture of the Circadian Clock (CC)-oscillator

The recruitment of BMAL1/ CLOCK-heterodimer to the E-Box DBS present in the promoter-enhancer elements of numerous CCGs, including *Periods* (*Per1/2*) and *Cryptochromes* (*Cry1/2*) augment their expression during the rest phase. Following accumulation, PERs and CRYs proteins dimerize and translocate to inhibit BMAL1/ CLOCK-dependent transcription during the active phase. Next, post-translational modifications including ubiquitination induce proteasomal degradation of PERs and CRYs, thus, initiating the next circadian cycle. In the second loop, BMAL1/CLOCK-dependent expression of *Rev-Erbα/β* during the rest phase, leads to the trans-repression of several RORE-DBS-containing CCGs including, *Bmal1*, *Clock* and *E4BP4*. In the active phase, the reduction in REV-ERBs levels permit the RORα/γ-dependent RORE-mediated activation of CCGs including *Bmal1* and *Clock*, which enables the turning of the circadian clock. Furthermore, DBP expression during the rest phase activates D-Box DBS containing CCGs, which are transcriptionally repressed by E4BP4 during the active phase. These coupled transcriptional-translational regulatory circuits are ubiquitously present in almost all cell types and directly control the expression of a vast number of mammalian genes.
CCG-Clock Controlled Genes. E-CCGs: E-Box DBS-containing CCGs, R-CCGs: RORE-containing CCGs, D-CCGs: D-Box-containing CCGs.

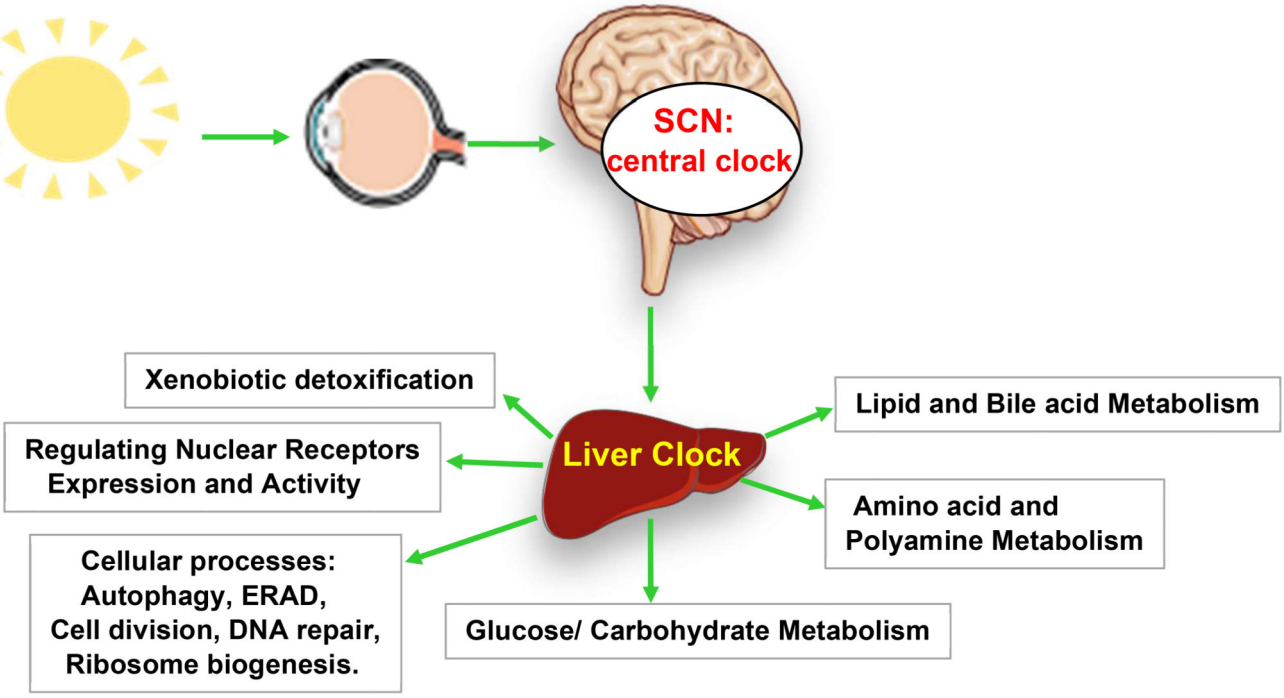

Figure 2. The clock controls the physiology of liver

Light-entrained central SCN-clock synchronizes peripheral tissue clocks including that of liver. The 'clock' machinery in turn drives the expression of several key transcription factors, rate limiting enzymes and transport proteins to spatiotemporally regulate several biochemical processes, which, together maintain physiological homeostasis. The 'clock'-connections to some of these processes and their connections to NAFLD and NASH have been discussed in detail.

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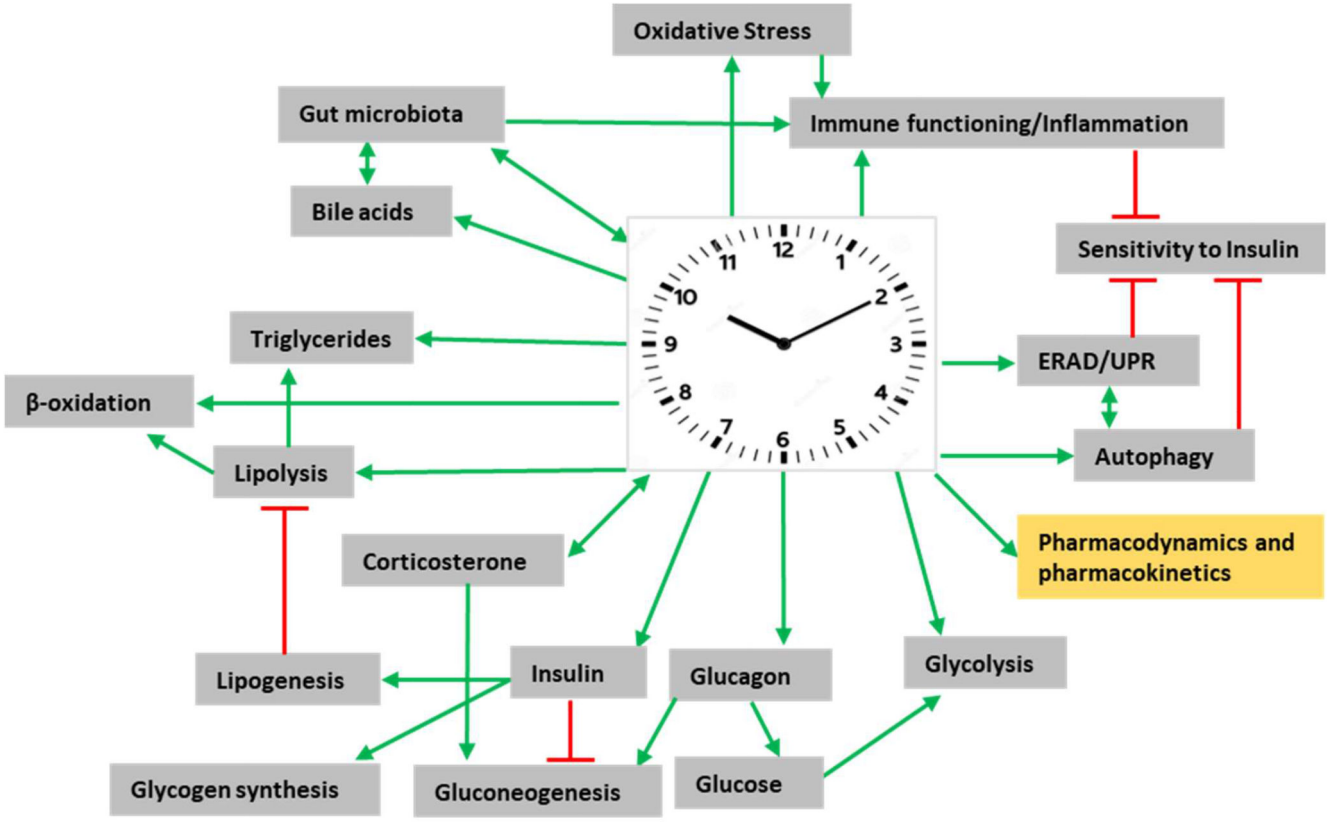

Figure 3. Multidimensional connections of the 'clock' to the pathogenesis of fatty liver

Model representing a global view of how alterations in circadian clock-controlled 'rhythmic' functions/pathways and processes could predispose to non-alcoholic fatty liver disease. Knowledge of the mechanisms through which the 'clock'-system influences all these systems and essential pharmacological parameters, in turn, could be utilized to develop novel chronotherapeutics. Green arrowheads represent activation and red bar-heads represent inhibition. See text for details.