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REVIEW ARTICLE

# Coupled network of the circadian clocks: a driving force of rhythmic physiology

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**The circadian system is composed of coupled endogenous oscillators that allow living beings, including humans, to anticipate and adapt to daily changes in their environment. In mammals, circadian clocks form a hierarchically organized network with a 'master clock' located in the suprachiasmatic nucleus of the hypothalamus, which ensures entrainment of subsidiary oscillators to environmental cycles. Robust rhythmicity of body clocks is indispensable for temporally coordinating organ functions, and the disruption or misalignment of circadian rhythms caused for instance by modern lifestyle is strongly associated with various widespread diseases. This review aims to provide a comprehensive overview of our current knowledge about the molecular architecture and system-level organization of mammalian circadian oscillators. Furthermore, we discuss the regulatory roles of peripheral clocks for cell and organ physiology and their implication in the temporal coordination of metabolism in human health and disease. Finally, we summarize methods for assessing circadian rhythmicity in humans.**

**Keywords:** circadian disruption; circadian misalignment; human circadian system; intercellular coupling; metabolic diseases; peripheral clocks; physiology and metabolism

## Molecular architecture of mammalian circadian systems

Virtually all living beings, including humans, possess an endogenous time-keeping system, composed of hierarchically organized body clocks, which governs biological and behavioral rhythms with a *ca.* 24-h period. Almost every cell in the body possesses the molecular machinery generating circadian oscillations. On the tissue and systemic level, single-cell oscillators synchronize with each other in order to maintain coherent network rhythmicity. Circadian rhythms are distinguished from other rhythmic biological processes by three defining properties: (a) They cycle with an endogenous free-running period of about 24 h, (b) they can entrain

**Abbreviations**
CBS, cystathionine β-synthase; ipRGCs, intrinsically photosensitive retinal ganglion cells; mTOR, mechanistic target of rapamycin; PPARγ, peroxisome proliferator-activated receptor-gamma; PRC, phase response curve; RHT, retinohypothalamic tract; SCN, suprachiasmatic nucleus; TTFLs, transcriptional–translational feedback loops; VIP, vasoactive intestinal peptide.



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to rhythmic environmental signals (Zeitgebers), and (c) their periods are temperature-compensated.

Evolutionary, circadian clocks are believed to have manifested because they provide intrinsic and extrinsic adaptive advantages. In particular, they allow organism to adapt to, as well as to anticipate daily environmental changes, while at the same time temporally coordinating incompatible endogenous processes (for review, see Ref. [1]). Field studies have shown that perturbation of the circadian system results in reduced survival due to increased vulnerability to predator attacks or mistiming of hibernation [2–5]. Moreover, laboratory species pass on circadian rhythmicity over many generations despite the absence of rhythmic environmental cues, suggesting that, indeed, circadian clocks provide an intrinsic advantage worth maintaining [6]. Thus, not surprisingly, both disruption of internal clock's synchrony and desynchronization between endogenous circadian and environmental Zeitgeber cycles are associated with various pathologies in humans. Here, we review fundamental design principles of the mammalian circadian systems and implications of circadian disruption for human health and disease. We further provide a glossary (Table 1), attempting to reduce sematic misunderstandings and to define terms commonly used in chronobiological research, especially when studying human circadian clock systems.

**Table 1.** Glossary of chronobiology.

| | |
|---|---|
| Actogram | Graphical representation of daily or circadian behavioral activity over several cycles |
| Amplitude | Peak-to-trough distance (absolute amplitude) or ratio (relative amplitude) of a rhythmic variable during a circadian cycle |
| Chronotype | Behavioral manifestation of the phase of entrainment reflecting the phase angle of an endogenous circadian variable (e.g., DLMO, biomarker expression, or sleep midpoint) with respect to a Zeitgeber (e.g., light onset) |
| Circadian | Lat. circa = about, dies = day |
| Circadian misalignment (also disruption) | Disturbance of the circadian system due to desynchronization with exogenous Zeitgeber cycles; may occur on organismal, system, tissue, and cellular levels |
| Circadian time (CT) | Time defined by the endogenous circadian period in constant conditions; circadian day = one complete circadian cycle |
| Constant conditions | Absence of rhythmic environmental cues (Zeitgebers) |
| Coupling | Mechanism, by which interacting oscillators cycle with stable phase and period relationships |
| Damping | Decline of the amplitude over time |
| Desynchronization | Process describing that previously synchronized oscillators gradually cease to cycle with stable phase and period relationships |
| DLMO | Dim light melatonin onset |
| Entrainment | (Period) synchronization of circadian oscillations to a rhythmic Zeitgeber leading to a stable phase relationship |
| Free-running period ($\tau$) | Endogenous circadian period (in constant conditions) |
| Masking | Acute response of an organism to an external Zeitgeber without involvement of the endogenous circadian system |
| MCTQ | Munich ChronoType Questionnaire |
| MEQ | Horne-Ostberg Morningness-Eveningness Questionnaire |
| MSF | Mid-sleep on free days: (Wakeup time – Sleep onset time)/2 |
| MSFsc | MSF corrected for sleep debt on workdays: MSF – (sleep duration on free days – average sleep duration)/2 |
| Period | Duration of a complete circadian cycle ($\tau$) |
| Phase | Reference time point of an oscillatory cycle, usually acrophase = peak time of a circadian variable |
| Phase of entrainment | Stable phase relationship between endogenous circadian and entraining Zeitgeber cycle |
| Phase response curve (PRC) | Graphical representation of phase shifts in response to Zeitgeber pulses over the course of a circadian day |
| Phase resetting | Zeitgeber stimulus-dependent shift of circadian oscillations, usually considered to induce synchronization of oscillator networks |
| Photoperiod | Duration of the light span within the course of the light–dark cycle |
| Range of entrainment | Range of permissible Zeitgeber periods an oscillator can entrain to (given a fixed Zeitgeber strength) |
| Resonance | Amplitude expansion of interacting weak oscillators when their periods approximate each other |
| Subjective day/night | Refers to times in constant conditions that correspond to day/night in a light–dark cycle |
| Zeitgeber ('time giver') | Rhythmic timing signal endogenous circadian oscillators can entrain to; cycles with the Zeitgeber period (T) |
| Zeitgeber time (ZT) | Time defined by the Zeitgeber cycle, commonly the light–dark cycle |

## Circadian organization at the system level

### The suprachiasmatic nucleus, pacemaker clock

Circadian rhythms are endogenously generated and not merely driven by environmental cycles: Oscillations persist even in the absence of external Zeitgeber cycles. However, life under natural conditions requires daily synchronization to rhythmic environmental signals in order to tune the period of circadian clocks to exactly 24 h, as well as to adapt circadian rhythms to seasonally changing photoperiods. Already in the 1960s, Colin Pittendrigh, one of the founding fathers of chronobiology, postulated that circadian systems are composed of a light-sensitive 'pacemaker' clock and subordinate oscillators in the periphery [7]. Today, we know that the suprachiasmatic nucleus (SCN), two bilateral neuron clusters located in the anterior hypothalamus, receives environmental photic information and subsequently synchronizes peripheral tissue clocks. Based on initial lesion and transplantation experiments demonstrating that the SCN governs behavioral and humoral rhythms with the period of the donor [8–11], the SCN has been proposed as a driver of circadian rhythms in mammals. As mammalian pacemaker clock, it ensures entrainment of the organism to the external light–dark cycle. Photic information is received by visual and non-visual photoreceptors of the retina and passed on the SCN *via* the retinohypothalamic tract (RHT). Entrainment signals appear to differ from visual information as the circadian system can respond to photic information despite visual blindness [12–14]. Melanopsin-expressing, intrinsically photosensitive retinal ganglion cells (ipRGCs) are crucial for mediating light-dependent phase resetting and entrainment [13,15–18]. Following photic entrainment, the SCN transmits time information to the periphery *via* direct efferent projections or indirectly *via* the regulation of hormone production and secretion, as well as of body temperature and behavioral cycles (for review, see Ref. [19]).

Within recent years, network topology and function of the SCN have become topics of increasing interest. It is known that the SCN of humans consists of roughly 100 000 neurons distributed across SCN core and shell regions, characterized by distinct afferent connections, neurotransmitter profiles, and phasing of circadian rhythms (for review, see Ref. [20]). However, the identity of distinct cell types and their distribution throughout the SCN, as well as their functional roles for circadian rhythm generation of the SCN as a whole, remain unclear. Recently, eight major cell types with distinct circadian gene expression patterns and light responsiveness have been identified by single-cell analysis of the SCN [21]. Night-active astrocytes rather than day-active neurons have been demonstrated to control and sustain molecular oscillations of the SCN and rhythmic behavior *via* glutamatergic signaling [22,23]. Moreover, it has been shown that the amplitude ratio between light-sensitive ventrolateral and light-insensitive dorsomedial regions determines the entrainment range of the SCN [24,25].

### Peripheral circadian clocks, slave oscillators

Almost 30 years following the discovery of the SCN, autonomous circadian rhythms were detected in nearly every tissue inside [26–28] and outside the brain [29–33]. Genome-wide transcriptome profiling showed that, depending on the tissue, 2–20% of genes are rhythmically regulated with little overlap between tissues [31,34–37]. This suggests that key physiological functions need to be temporally coordinated in order for organs to fulfill their biological tasks properly. Such functions include wound healing [38], detoxification [39], female reproduction [40], blood pressure and heart rate regulation [41], immune function [42], as well as carbohydrate and lipid metabolism [43–45] (for review, see Ref. [46,47]). However, despite tissue-specific circadian rhythmicity in the periphery, *ex vivo* and *in vivo* experiments suggest that the SCN is required to maintain correct phase relationships among body clocks [29,48]. Thus, peripheral oscillators are commonly considered as slave oscillators, which require orchestration by the pacemaker clock.

Besides SCN-derived signals, a multitude of exogenous signals can phase-shift peripheral circadian oscillators. Strong external Zeitgebers, predominantly feeding cues, presented in antiphase to the usual feeding–fasting cycle may even induce desynchronization between the SCN pacemaker and peripheral clocks [49–52]. Entrainment of peripheral clocks to feeding–fasting cycles may depend on hormone signals upon feeding. Especially, the liver, as major metabolic organ, has been suggested to act as mediator of food-driven entrainment of other peripheral oscillators. Recently, angiopoietin-like 8 (*Angptl8*) and tight junction protein 1 (TJP1) have been reported to regulate liver clocks in response to food by altering expression levels and activity of the clock gene *Per1* [53,54]. Subsequently, the liver may feed back to other body clocks *via* hormonal or metabolic routes. In addition, glucocorticoids have been demonstrated to serve as potent Zeitgeber signals for peripheral circadian clocks. Rhythms in glucocorticoids, controlled by SCN-derived signals and autonomous adrenal clocks, as well as bursts of glucocorticoids, induced by stress

or exercise, feed back to a number of peripheral and brain circadian oscillators [55–57]. Since the SCN does not express glucocorticoid receptors [58,59], glucocorticoids act as specific entrainment signals for extra-SCN brain, as well as peripheral circadian oscillators *in vitro* and *in vivo* [60,61]. Recently, the independence of peripheral tissue clocks from other body clocks has attracted attention. New mouse models allowed studying skin and liver oscillations in otherwise clock-less and thus behaviorally arrhythmic animals and have yielded indications of sustained rhythmicity within these tissues [44,62]. Such studies will lead to a better understanding of how various body clocks interact and may induce a paradigm shift redefining the role of peripheral clocks within mammalian circadian systems.

## Entrainment

### Conceptual background

In addition to seasonal changes in the temperature and light–dark cycle, modern lifestyle is often accompanied by large variations in environmental conditions, that is, artificial lightening, shift work, physical activity, mealtimes, and travel across time zones. Entrainment ensures daily alignment of endogenous circadian rhythms with environmental cycles within permissible period ranges [63]. The so-called range of entrainment is defined by the period limits to which the endogenous circadian system can still entrain. Entrainment range depends on the robustness of the intrinsic circadian oscillator, that is, its amplitude and relaxation rate following perturbation, as well as the strength of the Zeitgeber [64,65]. Therefore, strong oscillators, like the SCN, display narrow, and weak oscillators, like peripheral clocks, display large entrainment ranges. For example, lung explant oscillations can entrain to 20- and 28-h temperature cycles (2 °C temperature change), while SCN oscillations cannot [64].

Within any given range of entrainment, the Zeitgeber cycle and endogenous circadian rhythm attain stable phase relationships, which may range between ±6 h [65,66]. The so-called phase of entrainment, also referred to as chronotype in the context of human behavior, is governed by the period difference between Zeitgeber cycle (e.g., the 24-h light–dark cycle) and the endogenous circadian cycle, as well as by the Zeitgeber strength relative to oscillator amplitude. This implies that for a given Zeitgeber, the phase of entrainment will approach +6 or −6 h as the period mismatch between exogenous and circadian cycle becomes larger. However, since permissible period differences are confined by the range of entrainment, strong oscillators (narrow entrainment range) are expected to display stronger shifts in the phase of entrainment for a given period mismatch than weak oscillators (broad entrainment range) [66]. Theoretical concepts of entrainment may explain how distinct body clocks react to SCN-derived as well as external entrainment signals in a tissue-specific fashion. On the organismal level, the phases of entrainment of vertebrates are highly variable compared to unicellular organisms, insects, and plants [67]. Interestingly, human populations display broad chronotype distributions even though under natural conditions both Zeitgeber and endogenous circadian periods are relatively stable [68]. Thus, the human circadian system likely constitutes a very strong oscillator in order to display large variations in the phases of entrainment (chronotype) in response to small mismatches between exogenous and endogenous period.

Besides rhythmic entrainment cues, Zeitgeber signals may also occur as pulses, for example, when turning on artificial light during the night. How circadian oscillators respond to such pulsatile signals and in a time-dependent manner can be represented by so-called phase response curves (PRCs). Based on the extent of the phase response to a Zeitgeber signal, PRCs are distinguished into type-1 and type-0 PRCs. Type-1 PRCs are characterized by relatively small shifts and gradual transitions between phase delays and phase advances, while type-0 PRCs show large phase responses resulting in abrupt switches between delaying and advancing part [69]. Due to their Zeitgeber- and tissue-specific profiles, PRCs can help to deduce information about temporal gating and underlying mechanisms of phase adjustment.

### Photic entrainment of the SCN

Intrinsically photosensitive retinal ganglion cells rather than classical photoreceptor cells have been identified as predominant mediators of photic entrainment in mammals [12–14]. These ganglion cells depolarize in response to 480 nm light and remain active even during prolonged exposure to bright illumination due to expression of the photopigment melanopsin, whose knockout results in altered light responsiveness in mice [16,17,70]. Moreover, ectopic expression of melanopsin renders even peripheral cells photosensitive and enables phase shifts of circadian oscillations in response to light [71,72]. ipRGCs project to SCN neurons *via* the RHT and release neurotransmitters at synaptic clefts upon photic stimulation. Glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) are neurotransmitters mediating photic resetting of the SCN. Release and activation of their

respective G protein-coupled receptors result in the rapid induction of immediate early genes including components of the molecular clock machinery [73,74]. Most importantly, $Ca^{2+}$ and cyclic AMP (cAMP)-dependent kinase pathways mediate photic resetting by inducing cAMP response element (CRE)-driven expression of *Per1* and *Per2* ([75]; for review, see Ref. [76]). In addition, $Ca^{2+}$ and Ras-dependent activation of MAP kinase (MAPK) pathways converge on the transcriptional induction of CRE and serum response element (SRE), another enhancer element driving the immediate early expression of clock genes [73,77]. Moreover, phosphorylation and activation of CRE element-binding proteins (CREBs) play an important role for mediating downstream effects of photic signals [78]. Recently, phosphorylation of CREB at Ser133, a residue involved in binding of its transcriptional coregulator CBP (CREB-binding protein), has been shown to be required for normal locomotor activity and entrainment behavior in mice [79]. Due to the rhythmic expression of clock genes, immediate early induction of molecular clock components constitutes a temporally gated input pathway to the central pacemaker, thereby mediating time-of-day-dependent light responses of the mammalian circadian system. Photic PRCs in mammals display phase delays in response to light stimulation during the early subjective night, phase advances during the late subjective night, and no phase responses during the subjective day. Interestingly, despite antiphasic locomotor activity rhythms, photic PRCs are similar for nocturnal and diurnal mammals. However, the underlying mechanisms of this activity switch remain unknown (for review, see Ref. [80]).

### Non-photic entrainment of peripheral clocks

In order to align the mammalian circadian system with the light–dark cycle and to coordinate biological rhythms of various body clocks, the SCN forwards timing information to the periphery. SCN outputs are manifold and include neuronal innervations, as well as regulation of endocrine signaling, body temperature, feeding, and behavior. Transplantation experiments with encapsulated SCN grafts have shown that efferent neuronal connections are not necessary to drive rhythmic locomotor activity [81], suggesting that paracrine molecules, secreted by the SCN, govern activity rhythms. Several rhythmically secreted candidate molecules have been proposed to be involved in the control of locomotor activity by the SCN, including cardiotrophin-like cytokine (CLC) [82], prokineticin-2 (PK2) [83,84], and transforming growth factor-alpha (TGF-α) [85]. Recently, neuronal/myocyte-specific enhancer factor 2D (MEF2D) was demonstrated to regulate free-running behavioral period without affecting SCN rhythmicity itself, suggesting that this transcription factor controls SCN output pathways linking to activity [86]. In turn, rhythmic behavior, driven by the SCN, translates into indirect entrainment cues for peripheral circadian clocks by regulating feeding–fasting and body temperature cycles. In addition to behavior, ambient temperature cycles are able to sustain and entrain peripheral tissue oscillations independently of the SCN *in vivo* [50,87]. Heat-shock factor 1 (HSF1) and cold-inducible RNA-binding protein (CIRBP) are involved in temperature entrainment and responses of peripheral clocks to temperature pulses [88–92].

Moreover, peripheral circadian clocks are exposed to SCN-independent internal and external Zeitgeber information. Feeding–fasting cycles are dominant Zeitgebers for peripheral circadian clocks. Under normal conditions, feeding–fasting and rest–activity cycles are oscillating in phase with each other. However, if both Zeitgebers are presented in antiphase, peripheral oscillators entrain to feeding rather than SCN-derived signals, leading to internal desynchrony between body clocks [49,51,52]. Interestingly, arrhythmic feeding has been demonstrated to disrupt rhythms in hepatic signaling and metabolic pathways without altering the core clock machinery [93], suggesting that feeding rather than cell-autonomous molecular clocks governs rhythms in liver functions. Precise mechanisms of food-dependent entrainment are still under investigation. However, feeding-associated hormones and metabolites, as well as metabolic and redox states, have been suggested to convey nutritional information to circadian clocks. For example, fluctuations in nicotinamide adenine dinucleotide ($NAD^+$) cofactors and endogenous $H_2O_2$ are sensed by the molecular clock machinery, thereby regulating circadian rhythmicity [94,95]. Moreover, the $NAD^+$ sensing protein deacetylase SIRT1 regulates the magnitude of clock gene expression in the periphery [96–98]. In addition to redox oscillations, insulin and gastrointestinal hormones can influence peripheral tissue clocks, in particular the liver clock (for review, see Ref. [99]). Insulin alters circadian rhythms by inducing protein kinase B (AKT), mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K) pathways [100,101]. Various gastrointestinal hormones are rhythmically secreted and can act as regulators of peripheral circadian clocks, including glucagon-like peptide 1 (GLP-1), vasoactive intestinal peptide (VIP), oxyntomodulin (OXM), gastrin, ghrelin, cholecystokinin

(CCK), and others [102–104]. Recently, the mechanistic target of rapamycin (mTOR) pathway has been implicated as important link between feeding, metabolic state, and peripheral circadian clock function [105,106].

Neuronal innervation by the SCN appears to be required for the control of peripheral oscillators *via* hormone and humoral systems [10,107,108]. The SCN forms efferent projections to other brain areas, which in turn regulate body clocks *via* the autonomic nervous system [109,110]. Importantly, the SCN pacemaker controls rhythmic glucocorticoid release from the adrenal gland *via* the hypothalamic–pituitary–adrenal axis (HPA axis) [111–113]. As mentioned before, glucocorticoid rhythms constitute one of the most potent entrainment signals for peripheral circadian clocks. Adrenalectomy was shown to attenuate amplitudes of clock gene expression in a number of peripheral tissues, including liver, kidney, visceral adipose tissue, and jejunum [114]. Independent of the SCN, the adrenal clock itself, as well as stress and exercise, can drive rhythms in glucocorticoid release *via* activation of the sympathetic nervous system (for review, see Ref. [115]). Both physical activity and stress are entrainment signals for peripheral clocks [57,116–119]. Besides glucocorticoid secretion, the SCN, *via* the autonomic nervous system, also controls rhythms in blood pressure, body temperature, glucose production and sensitivity, feeding and drinking behavior, and the female reproductive cycle (for review, see Ref. [120]).

### Feedback to the SCN

In a complex network like the mammalian circadian system, it appears likely that systemic and mutual feedback regulations adjust circadian rhythms of body clocks. While precise mechanisms are still under investigation, SCN neuronal activity has been demonstrated to be altered by feeding-dependent signals, for example, ghrelin and leptin [121,122], blood pressure [123], or the immune system [124]. Adipocyte circadian clocks, *via* an adipocyte–hypothalamic axis and fatty acid secretion, have been suggested to be involved in the regulation of feeding–fasting cycles [125]. Moreover, cancerous tissues seem to be able to alter the molecular clock machinery in remote healthy tissues [126–128]. Recently, and with the help of a newly developed mouse model expressing functional clockwork only within a specific peripheral tissue, it was shown that liver and skin clocks depend on feedback of other body clocks in order to express their full circadian function [44,62].

## Cell-autonomous circadian clocks

Until the 1970s, the underlying molecular mechanisms of circadian rhythm generation remained unknown. However, with the discovery of so-called clock genes driving oscillations and determining circadian period [129–132] a common molecular design principle of circadian clocks started to emerge: Nearly all tissues express the molecular clock machinery that generates cell-autonomous, endogenous, and self-sustained oscillations [133–135].

### Molecular clock machinery

Circadian oscillations are generated by intertwined transcriptional–translational feedback loops (TTFLs) between genes and their protein products (Fig. 1). The molecular clockwork within SCN and non-SCN tissues is nearly identical consisting of the same clock genes and proteins driving oscillations *via* positive and negative TTFLs. In brief, three interlocked delayed feedback loops generate circadian biological rhythms. In the so-called core loop, BMAL1 and CLOCK proteins form a heterodimeric transcription factor that drives the expression of its target genes *via* the activation of E-box enhancer elements. After a defined time delay, important for the generation of *ca*. 24-h rhythms [136], protein products of CLOCK/BMAL1 target genes, such as *Period* (*Per1,2,3*) and *Cryptochrome* (*Cry1,2*) genes, translocate back into the nucleus where they repress their own transcription. For many years, CRY and PER proteins have been assumed to function as heterodimers; however, evidence for their association with large macromolecular protein assemblies, including all isoforms of PER and CRY, as well as casein kinase 1δ (CK1δ), has been accumulated over the last years ([137]; for review, see Ref. [138]). In two additional feedback loops, CLOCK/BMAL1 transcription factor drives the expression of the retinoic acid-related orphan nuclear receptor *Rev-erbα/β*, the RAR-related orphan receptor *Rorα/β,* as well as the D-site albumin promoter binding protein *Dbp* [139]. REV-ERBs and RORs competitively regulate the expression of the core loop component *Bmal1 via* RORE enhancer elements, whereas DBP (as transcriptional activator) and its opponent E4BP4 (as transcriptional repressor) compete for binding to D-box sequences in the promoters of their target genes *Rev-erbα/β*, *Ror/β*, *Per1,2,3,* and *Cry1*. While the core feedback loop is required for rhythm generation *per se*, the additional loops have been proposed to play a role for fine-tuning of period, phase, and amplitude of circadian oscillations [136,140]. Moreover, clock transcription factors drive

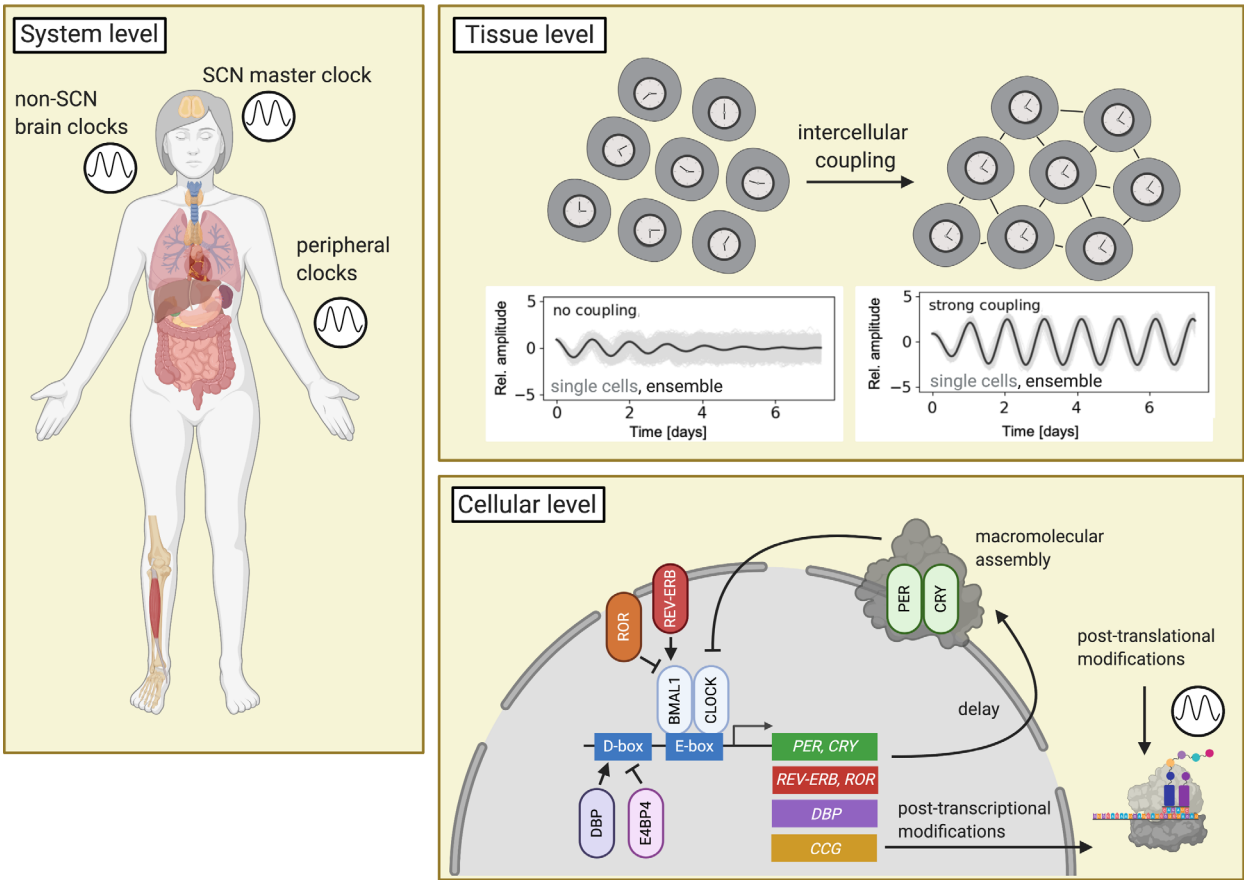


**Fig. 1.** Organizational levels of mammalian circadian systems. Mammalian circadian clocks consist of cell-autonomous and self-sustained oscillators that can be found in virtually all cell types. At the system level, a 'master clock' located in the SCN of the hypothalamus ensures entrainment of subsidiary peripheral oscillators to the light–dark cycle, as well as alignment of body clocks with each other. At the tissue level, rhythmicity is generated by ensembles of cell-autonomous oscillators. Since single-cell oscillators cycle with their own periods and phases, desynchronization, caused by progressive dephasing, can be avoided by intercellular coupling. At the cellular level, circadian oscillations are driven by interconnected feedback loops between core clock genes and their protein products. Tissue-specific circadian outputs are generated by the combined effects of rhythmic transcriptional, post-transcriptional, translational, and post-translational processes (CCE, clock-controlled enhancer element; CCG, clock-controlled gene). This figure was created with BioRender.

the expression of clock-controlled output genes (CCGs) in a time- and tissue-specific fashion. Thus, it is not surprising that *ca.* 5–20% of transcripts, proteins, and metabolites exhibit circadian rhythms [34–36,141–144].

Although clock genes are constitutively expressed at specific developmental stages, circadian oscillations have not been detected in germline cells, zygotes, early embryos, as well as embryonic and induced pluripotent stem cells [145–149]. Nevertheless, circadian rhythms gradually and cell-autonomously emerge during development when cells start differentiating [148,149]. Reprogramming and disturbance of cellular differentiation perturb the development of circadian oscillations, suggesting that both biological processes are interconnected [148]. Nevertheless, precise mechanisms remain unknown. Importin subunit alpha (*Knap2*) has been described to be important for the development of circadian rhythms by regulating PER2 subcellular localization [150]. More recently, post-transcriptional suppression of CLOCK, potentially *via* the endonuclease–microprocessor complex *Dicer*/*Dgcr8*, has been suggested to regulate circadian clock development [151,152].

## Non-TTFL rhythm generation

In addition to TTFLs, post-transcriptional, post-translational, and non-transcriptional mechanisms have been described to regulate or even drive circadian oscillations on the cellular level. Surprisingly, only 20–30% of rhythmic mRNA transcripts appear to depend

on cyclic de novo transcription [153], suggesting an important role of post-transcriptional regulation for circadian rhythm generation (for review, see Ref. [154]). For example, (a) mRNA stability of the clock components *Cry* and *Per* has been demonstrated to vary throughout the day due to 3′-UTR and poly(A)-tail regulation [155–157], (b) miRNAs have been suggested to regulate circadian rhythms by targeting clock or clock-controlled genes [158,159], and (c) nuclear export dynamics of clock mRNAs has been indicated in period determination of circadian rhythms [92,160].

Within recent years, advances in mass spectrometry and associated bioinformatics analysis have enabled large-scale proteomic studies, also on the level of post-translational modifications. Only up to 50% of rhythmic RNA transcripts translate into rhythmically expressed proteins [141,142,161], further supporting the importance of post-transcriptional or even post-translational mechanisms for circadian rhythm generation (for review, see Ref. [162]). One of the first single-gene mutations identified as circadian clock modulator, the *tau* mutation in golden hamsters, constitutes a missense mutation in the phosphate recognition domain of casein kinase 1ε [163], which targets many clock proteins. Phosphorylation is the most prevalent post-translational modification, and *ca.* 25% of phosphorylation sites oscillate in mouse liver [164,165]. Phosphorylation sites of core clock proteins have been found to be rhythmically modified and to regulate protein activity [100,164,166]. In addition to phosphorylation, other post-translational modifications can modulate circadian rhythmicity, including F-box/LRR-repeat protein (FBXL)-dependent ubiquitination of CRY proteins [167–170], sirtuin-1 (SIRT1)-dependent deacetylation of PER2 and BMAL1 [96,98,171], or SUMOylation of BMAL1 [172].

Lastly, redox cycles have been proposed to promote or even drive circadian rhythms independently of transcription and translation [173]. Peroxiredoxins (PRX) appear to display *ca.* 24-h oxidation cycles that persist under constant conditions, and are entrainable and temperature-compensated [174]. PRX are highly conserved antioxidant proteins required for the maintenance of cellular redox homeostasis. Potentially, transcriptional and redox cycles cooperatively regulate circadian rhythms; however, precise mechanisms of such a coupling remain elusive. Recently, endogenous $H_2O_2$ has been shown to exhibit circadian oscillations governing rhythmic oxidation of CLOCK proteins, as well as circadian dynamics in mice [95]. Moreover, PRX may be involved in circadian rhythm maintenance in liver and skin of *Bmal1*-deficient (behaviorally arrhythmic) mice [175].

## Oscillator coupling

Virtually, every tissue in the human body is composed of self-sustained and cell-autonomous circadian oscillators, which display a normal distribution of periods and phases across cellular populations ranging from about 20 to 28 h. If individual cells would cycle independently of each other and with their own period, phases of cellular oscillators would drift apart leading to desynchronized tissue rhythms over time (Fig. 1). Thus, single-cell oscillators within central and peripheral tissue clocks, for example, the SCN or the liver, need to either couple with each other or be synchronized to external or systemic Zeitgebers in order to maintain synchronized network rhythms.

Coupling serves to phase- and period-lock individual oscillators to maintain synchronized rhythms on the population level (Fig. 1). Without coupling, additional extrinsic or intrinsic Zeitgeber signals are required to synchronize cell-autonomous oscillators and generate coherent network oscillations; otherwise, period differences will result in oscillator desynchronization over time (Fig. 1). In 2018, Schmal *et al.* [176] mathematically defined three qualitative coupling states based on distributions of periods, phases, and amplitudes observable for oscillator networks. According to their study, coupling strength can be inferred from period and phase distributions. Coupled oscillators have more similar periods and phases and display larger amplitudes. This is the case because (a) coupled oscillators exert mutual phase- and period-pulling effects on neighboring oscillators resulting in convergence of these circadian parameters, and (b) resonance occurs among low-amplitude oscillators with similar periods leading to increased amplitudes (comparable to resonance effects in physics).

Intuitively, coupling is expected to result in reduced damping of ensemble rhythms since damping is caused by desynchronization of self-sustained oscillators. Moreover, Abraham *et al.* [64] reported that amplitude relaxation of coupled networks, that is, how quickly the population rhythm returns to its initial state following perturbation, accelerates with increasing coupling strength, indicating that coupled networks are more robust. As described above, entrainment properties are constrained by the robustness of the entrained oscillator. Therefore, interoscillator coupling, by promoting amplitude expansion and faster amplitude relaxation, constitutes an important determinant of the phase of entrainment, of the range of entrainment, and of the response to Zeitgeber signals. For example, more strongly coupled oscillator networks, like SCN tissue, are more difficult to entrain and more robust

against perturbation by Zeitgeber pulses than weakly coupled networks, like lung tissue [64].

In summary, intercellular coupling constitutes an integral feature of circadian clock systems, governing response to Zeitgeber signals and entrainment. Considering that body clocks are constantly exposed to a variety of extrinsic and intrinsic Zeitgeber signals that must be properly integrated to generate tissue-specific circadian outputs, coupling likely plays an important role for the correct timing of rhythmic biological functions. Studying coupling among circadian oscillators on both tissue and system levels increases knowledge about the multidimensional circadian clock system in mammals and improves the understanding of mechanisms leading to circadian disruption and associated pathologies.

## Coupling among cell-autonomous oscillators

Both SCN neurons and peripheral cells exhibit self-sustained circadian oscillations [133,134,177]. When cultured as nearly intact tissue explants, SCN neurons display narrow distributions of periods and phases as well as highly synchronized network oscillations with periods close to the behavioral periods [178]. Peripheral tissue slice cultures display persistent, yet dampened circadian rhythms for many days in culture [29], indicating that cell-autonomous oscillators remain synchronized with each other at least to some extent. Upon dissociation, SCN neurons as well as fibroblasts (a model for peripheral oscillators) exhibit desynchronized circadian oscillations with a broad period distribution [134,179]. Circadian rhythmicity of SCN neurons and fibroblasts is attenuated under sparse culture conditions [179,180]. Application of conditioned medium from densely cultured fibroblasts can rescue weak rhythmicity of sparse cultures [179]. Moreover, inhibition of interneuron communication by tetrodotoxin decreases rhythm amplitude of the SCN pacemaker as well as induces desynchronization of neuronal oscillators [181]. Together, these findings suggest that cell-autonomous circadian oscillators couple with each other *via* intercellular communication pathways. In addition, network interactions seem to promote robust circadian tissue rhythms. Nevertheless, while coupling on the tissue level is well-characterized for neuronal oscillators within the SCN, coupling within peripheral clock networks is still debated.

### Coupling within the SCN

Coupling among SCN neurons is strong enough to maintain robust network rhythms over long durations of time and even if single-cell oscillators are dysfunctional [182,183]. Spatiotemporal regulation of circadian dynamics across the SCN is complex with wave-like spreading of phases and amplitudes across different regions [181,184]. Thus, it appears that intercellular coupling within the SCN depends on defined neuronal circuits establishing interactions between distinct SCN regions in a temporally controlled manner (for review, see Ref. [185]). Interestingly, neuronal coupling in neonatal SCN was reported to be stronger than in adult SCN [186,187], suggesting that neuronal connections or involved neurotransmitters change throughout development. Moreover, SCN coupling is altered by abnormal lighting regimes, for example, during jetlag, leading to changes in the distribution of phases and neuronal firing rhythms (for review, see Ref. [185]). Eventually, such light-induced 'decoupling' may be beneficial to enhance plasticity of the circadian system allowing for faster entrainment to new light–dark cycles. Chronic perturbation of SCN coupling, however, for example, during social jetlag or shift work, may promote circadian disruption and associated pathologies. Synaptic release of neurotransmitters [181,188,189], unknown paracrine communication pathways [186,190], and direct communication *via* gap junctions [191–193] are believed to mediate synchronization among SCN neurons. Within the photic input receiving core region, VIP is the most abundant neurotransmitter. VIP is released rhythmically from SCN core neurons and binds to its respective receptor (VPAC2) in both core and shell SCN regions [194,195]. Functional studies have shown that VIP plays an important role in interneuron coupling and regulation of the behavioral period. Depletion of VIP or VPAC2 alters excitability and firing of SCN neurons [196,197], attenuates clock gene rhythms [187,198,199], and results in desynchronization of cell-autonomous oscillators [197,199,200]. Knockout of VIP or VPAC2 disrupts rhythms of locomotor activity and entrainment in mice [198,200,201], further suggesting that intercellular coupling is crucial for regulating rhythmic biological processes on the organismal level. In addition to VIP, other neurotransmitters such as vasopressin (AVP), gamma-aminobutyric acid (GABA), and gastrin-releasing peptide (GRP) have been described to modulate synaptic SCN coupling and activity rhythms in mice (for review, see Ref. [202]).

### Coupling within peripheral tissues

In contrast to the SCN, peripheral clock rhythms have been found to quickly dampen *in vitro* [32–34,203].

Some studies have reported that fibroblasts do not display signs of intercellular coupling [133,135]. Moreover, clock gene mutations, which can be compensated by coupling in the SCN, have been shown to disrupt tissue rhythms in the periphery [183]. However, as mentioned above, *ex vivo* slice cultures of peripheral tissue clocks, although with some degree of damping, have been found to display persistent network rhythms for many days [29]. *In vivo*, non-SCN clocks have been demonstrated to sustain coherent tissue rhythms independently of the SCN or rhythmic external Zeitgebers [48,204,205]. This suggests that cell-autonomous oscillators stay at least partially synchronized, as long as tissue integrity is intact. Moreover, mathematical models identified weak, intercellular coupling among peripheral oscillator models *in vitro*. Clusters of neighboring hepatocytes display more narrow phase and period distributions than distant neighbors or cells from uncoupled networks [206]. Fibroblasts exhibit slight phase-pulling effects on adjacent cells, although too weak to maintain synchronized network rhythms given the variability of endogenous periods [207]. Moreover, weak rhythmicity of sparsely cultured fibroblasts can be rescued by treating cells with conditioned medium from densely cultured cells [179], indicating that peripheral oscillators enhance network rhythmicity by exchanging paracrine signals. Interestingly, many proteins, including secreted ones, appear to be rhythmically controlled with respect to expression and secretion [142,208]. Although paracrine communication pathways likely contribute to intercellular coupling in the periphery, no mechanism has been identified so far. Therefore, coupling of cellular clocks within peripheral tissues remains in dispute. One may speculate that coupling within peripheral tissues is not required since, under normal conditions, peripheral clocks are exposed to strong synchronizing signals originating from the SCN or the environment. However, a correct temporal coordination of rhythmic biological functions appears to be crucial for an organism's well-being. Thus, intercellular coupling may be very important for the maintenance of peripheral rhythms in the absence of SCN-derived or external Zeitgeber, proper entrainment, and robustness toward acute Zeitgeber signals.

### Coupling among body clocks

Coupling within the circadian system may take place on different organizational levels (for review, see Refs [209,210]). Whether it occurs at the system level is still an open question. Since the SCN is the master pacemaker keeping body clocks in synchrony, the relevance of systemic coupling may not be apparent. Nevertheless, mutual exchange of time information among different body clocks may provide synchronized feedback from the periphery to the SCN or regulate organismic responses to external entrainment signals. Several communication pathways among body clocks have been described (for review, see Ref. [211]). Yet, it remains to be explored whether these pathways enable bidirectional coupling rather than unidirectional clock entrainment/resetting. Potentially, adjustment of the mammalian clock system to feeding signals or to energetic alterations constitutes a systemic coupling pathway (for review, see Ref. [212]). Feeding–fasting cycles act as dominant Zeitgebers for peripheral clocks [49,51] but alter SCN activity, body temperature, and rest–activity cycles *via* the release of feeding-related hormones [213–216], which again leads to feedback regulations of peripheral oscillators through neuronal routes.

Recently developed mouse models aim at investigating interactions of various body clocks. In 2019, two groups demonstrated that isolated peripheral tissue clocks maintain—at least in part—rhythmic gene, protein, and metabolite expression under light–dark cycles [44,62]. In constant darkness, however, tissue rhythms appeared to be lost, suggesting that (a) rhythmic light input is important to partially maintain tissue oscillations, but more importantly, that (b) full circadian function of peripheral clocks requires rhythmic input from other body clocks. Mouse models with various combinations of functional body clocks will help to elucidate systemic communication or coupling pathways within the mammalian circadian clock network.

## Peripheral clocks control cell and organ physiology: lessons from rodent studies

Coupling of myriads of individual tissue clocks across the body into a unified network, re-adjusted on the daily basis by the master pacemaker, ensures temporal coordination of physiology and metabolism [212,217–221]. Indeed, peripheral clocks operative in the organs play an essential role in temporal coordination of metabolic reactions, from food processing to xenobiotic detoxification, by ensuring anticipation of rest–activity cycles (reviewed in Refs [47,212,218,220,222–228]). Large-scale transcriptomic studies indicate that a significant fraction of the transcripts in liver, skeletal muscle, white adipose tissue (WAT), and endocrine pancreas is rhythmic in rodents [34,37,45,227,229–240]. In line with the transcriptomic data, proteins [142,161], microRNAs [241,242], free fatty acids (FFAs), lipids,

and other metabolites [44,243–245] are subject to circadian variations. Noteworthy, the oscillatory pattern of certain proteins and lipid metabolites was found to persist in arrhythmic mice kept in constant darkness under normal feeding–fasting cycles [161,243], suggesting that rhythmic feeding is sufficient to drive a subset of proteins/metabolites independent of the endogenous clock machinery.

Concordantly with reported diurnal rhythmicity of genes, proteins, and metabolites, physiological outputs of the organs exhibit circadian variations. Indeed, detoxification and metabolic function of the liver, response to insulin by skeletal muscle or WAT, as well as secretion of hormones and cytokines by pancreatic islet α- and β-cells and by intestinal L cells exhibit diurnal rhythmicity [45,125,231,233,234,246–253]. As mentioned above, some rhythmic liver functions appear to depend on the presence of other body clocks, while others, like carbohydrate, amino acid, and redox-related metabolic processes, are exclusively driven by liver autonomous circadian clocks [44]. Taken together, these studies provide compelling evidence for the circadian system temporally coordinating metabolic physiology. Consistently, genetic disruption of the core clock components leads to perturbations of carbohydrate, lipid, and protein metabolism, and such mouse models develop hyperphagia, obesity, hyperglycemia, and glucose intolerance [45,125,233,239,250,251,254–257].

Whereas whole-body BMAL1-KO animals develop various pathologies, including hyperglycemia, hyperlipidemia, and premature aging [258], tissue-specific knockout models of the key core clock element BMAL1 exhibit distinct metabolic phenotypes. Liver-specific BMAL1-KO mice are hypoglycemic [43]. They display reduced lipid accumulation *via* increased mRNA methylation, in particular of PPARα [259,260], and alterations in the hedgehog pathway leading to the steatosis development [249]. Muscle-specific BMAL1-KO leads to metabolic inefficiency and impairs muscle triacylglycerol biosynthesis [261]. BMAL1 directly regulates *Myod*, a master regulator gene in the skeletal muscle [37,236]. In turn, MYOD binds to enhancer elements in the *Bmal1* promotor and acts synergistically with BMAL1/CLOCK to regulate clock-controlled genes in the skeletal muscle [262]. Pancreatic islet-specific BMAL1-KO leads to development of overt type 2 diabetes (T2D) [45], also when it is induced in the adult age [233], and it reduces the metabolic adaptation to HFD-induced obesity [254]. Finally, animals bearing an adipocyte-specific BMAL1-KO become obese [125].

In addition to BMAL1-KO, mice with disrupted nuclear receptor REV-ERBα exhibit perturbed lipid and carbohydrate metabolism [263–269]. In the liver, REV-ERBα couples glucocorticoid signaling to energy metabolism *via* binding of the hepatocyte nuclear transcription factors HNF4A/HNF6 [270]. Additionally, ROR nuclear receptors were reported to enhance mitochondrial respiration and ATP production in skeletal muscle, *via* transcriptional activation of the key regulator genes [271]. Based on this conjunction, ROR agonist nobiletin promoted healthy aging in mice subjected to the high-fat diet regimen [271]. The core clock component PER2, element of the negative feedback limb, coordinates lipid metabolism by regulating key enzymes in the lipid biosynthesis and peroxisome proliferator-activated receptor-gamma (PPARγ) [243,272,273]. PPARγ signaling was also perturbed in the WAT of mice deficient in the transcriptional repressor DEC1, leading to disturbance of lipid turnover in WAT and to disrupted rhythmicity of FFAs in serum [274]. Hepatic overexpression of another negative regulator, CRY1, inhibits glucagon-induced liver gluconeogenesis, leading to lowered blood glucose levels and improved insulin sensitivity in the insulin-resistant *db*/*db* mice [250]. Furthermore, CRY1 levels are decreased by autophagy in rodent liver [275]. In CRY1-KO mice, reduced cystathionine β-synthase (CBS) activity levels were reported that were rescued by adding exogenous CRY1. CRY1-induced CBS activation led to post-translational switch that modulated metabolism [276]. Interestingly, the circadian regulator Nocturnin, a rhythmic gene encoding a deadenylase thought to be involved in the removal of poly(A) tails, controls glucose and lipid metabolism [277], as well as metabolic adaptation in brown adipose tissue [278]. Another RNA-binding protein, NONO, couples the rhythmic expression of metabolic genes in the liver with nutrient levels. Its genetic disruption leads to impaired glucose tolerance, lower hepatic glycogen, and decreased lipid content [279].

In summary, these findings strongly support the crucial importance of functional tissue clocks for cell and organ physiology, suggesting that disruption of only a single clock gene/protein can be associated with adverse health effects. However, when interpreting the impact of core clock transcriptional factors on metabolic outputs stemming from the studies in genetic mouse models of clock component disruption, one must recognize that these transcriptional regulatory proteins may also exert non-circadian functions. Indeed, whereas BMAL1-KO mice are prone to early aging [258], this is not the case for CLOCK mutant, as well as PER1/2- or CRY1/2-double KO animals. This might be explained by BMAL1's inhibitory effect on the mTORC1 pathway [280], resulting in increased

mTORC1 activity in BMAL1-KO. Thus, premature aging in BMAL1-KO is likely to be attributed to the non-circadian function of BMAL1 rather than to its role in the core clock machinery.

The connection between the circadian system and rhythmic physiology is reciprocal, with diurnal physiological alterations readjusting the peripheral oscillators on the daily basis. Indeed, feeding–fasting and temperature cycles, exercise, levels of oxygen, $CO_2$, and metabolites represent potent Zeitgebers for peripheral clocks (reviewed in detail in Refs [47,281]). At the cellular level, the core clock components can be adjusted by the local concentrations of metabolites. Nicotinamide adenine dinucleotides (NADs) affect the activity of core clock components directly, or *via* NAD-dependent enzymes, thus playing an essential role in fine-tuning of the molecular clock to the metabolic state [96–98,282–284]. Precursor of $NAD^+$, nicotinamide riboside (NR), increases BMAL1 chromatin binding *via* PER2 deacetylation that primes PER2 phosphorylation. This mechanism underlies beneficial effects of NR that rescues dampened oscillations of gene transcription and mitochondrial respiration in aged mice. Thus, $NAD^+$ drives reprogramming of metabolic and stress–response pathways that decline with aging [284,285].

## Approaches for studying human circadian clocks

Human circadian clocks drive rhythmic biological processes that govern organ functions, metabolism, and physiology, as well as behavioral rhythms such as sleep–wake and feeding–fasting cycles. Inter- and intra-individual differences in the endogenous rhythms determine how humans entrain to periodically reoccurring environmental conditions. This results in a wide range of chronotypes, that is, phase relationships between endogenous circadian and exogenous Zeitgeber cycles. People with short free-running periods are more likely to be early chronotypes ('morning larks'), while people with long free-running periods are more often late chronotypes ('night owls') when entrained to the daily light–dark cycle [286,287]. When Zeitgeber cycles are out of synchrony with the endogenous circadian cycle, for example, upon shift work, travel across time zones, social jetlag, or artificial lighting, serious health consequences may develop (for review, see Ref. [224]). Thus, studying circadian rhythms in humans in normal,, as well as under varying environmental conditions helps to uncover causes for circadian misalignment and associated pathologies. In addition, the endogenous circadian clock governs kinetics and dynamics of many, especially short-lived drugs, as well as outcomes of medical interventions or following injury [38,288,289]. The goal of adapting treatment times to endogenous circadian rhythms is to maximize therapeutic responses while minimizing side effects leading to an emerging field of medical research (chronomedicine, chronotherapeutics, and chronopharmacology) (for review, see Ref. [290,291]). However, treatment of patients during the 'right time of day' requires knowledge about the status of their individual circadian clocks, for example, their chronotype. Thus, studying human circadian clocks *in vivo* and *in vitro* has become a topic of increasing interest.

### Studying circadian clocks *in vivo*

The SCN is located deep in the hypothalamus; thus, quantification of the pacemaker oscillations in humans is only possible by observing rhythmic outputs driven by the underlying circadian system. Most commonly, these outputs include periodic variations in activity, sleep, body temperature, and hormone levels. In pioneering studies, Jürgen Aschoff and others uncovered the endogenous nature of the human circadian clock by isolating individuals from their rhythmic environments, while continuously measuring physiological and behavioral parameters. Rhythms of peripheral clocks may be quantified *in vitro* by sampling, for example, blood, skin, saliva, hair, oral mucosa, WAT, muscle, or urine in regular intervals over the course of a day. However, elaborated, quick, and sensitive molecular techniques for sample analysis are just now starting to be developed. Therefore, laboratory protocols are often still considered the gold standard for assessing the endogenous human circadian rhythms *in vivo*.

#### Chronotype (phase of entrainment)

Typically, and most simply, chronotype is estimated using questionnaires such as the MEQ or the MCTQ. While the MEQ is a self-assessment test scoring whether subjects feel more active/alert during the morning, the evening, or in between, the MCTQ asks for sleep timing during workdays and free days ([292,293]; for review, see Ref. [294]). Self-reported average mid-sleep time (MSF) or MSF corrected for sleep debt during workdays ($MFS_{sc}$) is assessed by the MTCQ and is commonly accepted as useful indicator of chronotype. MCTQ analysis of a large cohort of subjects suggests that chronotypes are roughly normally distributed across populations from different geographical areas. 2.5% of individuals at either end

of the chronotype distribution were classified as extreme morning types with $MSF/MSF_{sc} < 2.17$ or extreme evening types with $MSF/MSF_{sc} > 7.25$ [295]. For the MEQ, individuals with scores above 58/86 are considered morning types, while individuals with scores below 41/86 are considered evening types.

As a more accurate readout of the phase of entrainment, melatonin levels can be measured from saliva, blood, or urine samples. Melatonin is a sleep–wake cycle regulating hormone rhythmically secreted by the pineal gland. Its secretion is controlled by the SCN and starts 2–3 h before habitual sleep time. Interestingly, melatonin levels are robust toward perturbation by extrinsic or intrinsic cues, except light exposure, which acutely suppresses melatonin secretion [296]. In healthy individuals, timing of the dim light melatonin onset (DLMO) accurately reflects human chronotype when measured under constant conditions (dim light and controlled posture) and is reasonably well correlated with $MSF/MSF_{sc}$ and MEQ score [297,298]. However, laboratory assessment protocols of DLMO, due to their 30-min sampling intervals in the early night and controlled conditions, are expensive, time-consuming, and unpleasant for subjects. Home assessment kits allow individuals to collect saliva samples conveniently at home and in dim light. Studies show that home and laboratory DLMO measures display good correlation if participants are compliant with instructions [299] or may vary by up to 90 min when compliance is not monitored [300].

To predict phase of entrainment (or chronotype), biomarker (BM) approaches that require only one or a few single time point measurement have recently been developed. Pioneering work was performed by Ueda *et al.* [301] by creating a 'molecular timetable' of mouse livers, that is, circadian expression profiles of more than 100 time-telling genes, which enables to predict endogenous clock time from a single sample. This idea was adopted in human studies using cycling metabolites in human blood samples taken at two antiphasic time points [302] or from circadian transcriptomes of peripheral blood mononuclear cells (PBMCs) [303,304]. Today, BM prediction of circadian clock time, with accuracy comparable to DLMO assessment, is even possible by measuring only a small set of time-telling genes from a single time point blood sample, irrespective of the sampling time [304,305].

Despite advances in chronotype assessment, some questions remain to be investigated. How does chronotype change under non-natural conditions, for example, upon circadian disruption (shift work and travel across time zones) or disease (sleep disorders and inflammation)? How stable are individuals' chronotypes over time? Can assays be developed that clearly distinguish between phase of the central clock and peripheral clocks to assess internal desynchronization? Circadian misalignment may be inferred from phase relationships between distinct phase measures ($MSF/MSF_{sc}$, DLMO, biomarkers from various tissues) or between predicted phase and external Zeitgeber cycles, for example, light–dark cycle (Fig. 2).

## Endogenous period

Intrinsic or free-running period of the endogenous clock system is only revealed in a non-entrained state. To assess this, individuals need to either stay in constant conditions (constant routine protocols) or in conditions, to which they cannot entrain (forced desynchrony protocols). Moreover, serial sampling for at least 24 h is required to quantify clock-driven rhythmicity, for example, of melatonin, cortisol, body temperature, or other biomarkers (Fig. 2). Constant routine protocols measure endogenous circadian oscillations independently of behavioral or exogenous influences, such as ambient temperature, light, meals, social cues, activity, sleep, or even posture. Subjects are kept in constant dim light, constant temperature, constant posture, without sleep, and with evenly distributed isocaloric snacks (for review, see Ref. [306]). Such laboratory protocols are supposed to reduce masking effects of environmental Zeitgebers. However, aftereffects from entrainment signals prior to the study or masking effects and phase shifts introduced by experimental procedures themselves may confound the assessment of the free-running period ([307]; for review, see Ref. [308]). During forced desynchrony protocols participants are subjected to either 28- or 20-h Zeitgeber cycles, usually light–dark cycles, to prevent entrainment of the endogenous clock system and to be able to record free-running periods of sleep–wake and body temperature cycles [309,310]. Both constant routine and forced desynchrony protocols are labor-intensive, costly, and time-consuming. Thus, even though these methods constitute the gold standard to assess the endogenous circadian period, they are impractical and do not reveal behavior of the circadian system under changing environmental conditions. Alternatively, serial BM sampling from blood, saliva, hair, or tissue biopsies can provide information about an individual's endogenous circadian period when samples are collected in regular intervals and under constant conditions (Fig. 2) (for review, see Refs [311,312]). One advantage of such sampling is that BM of peripheral tissue clocks is likely insensitive to direct light exposure (in contrast to melatonin). However, for some

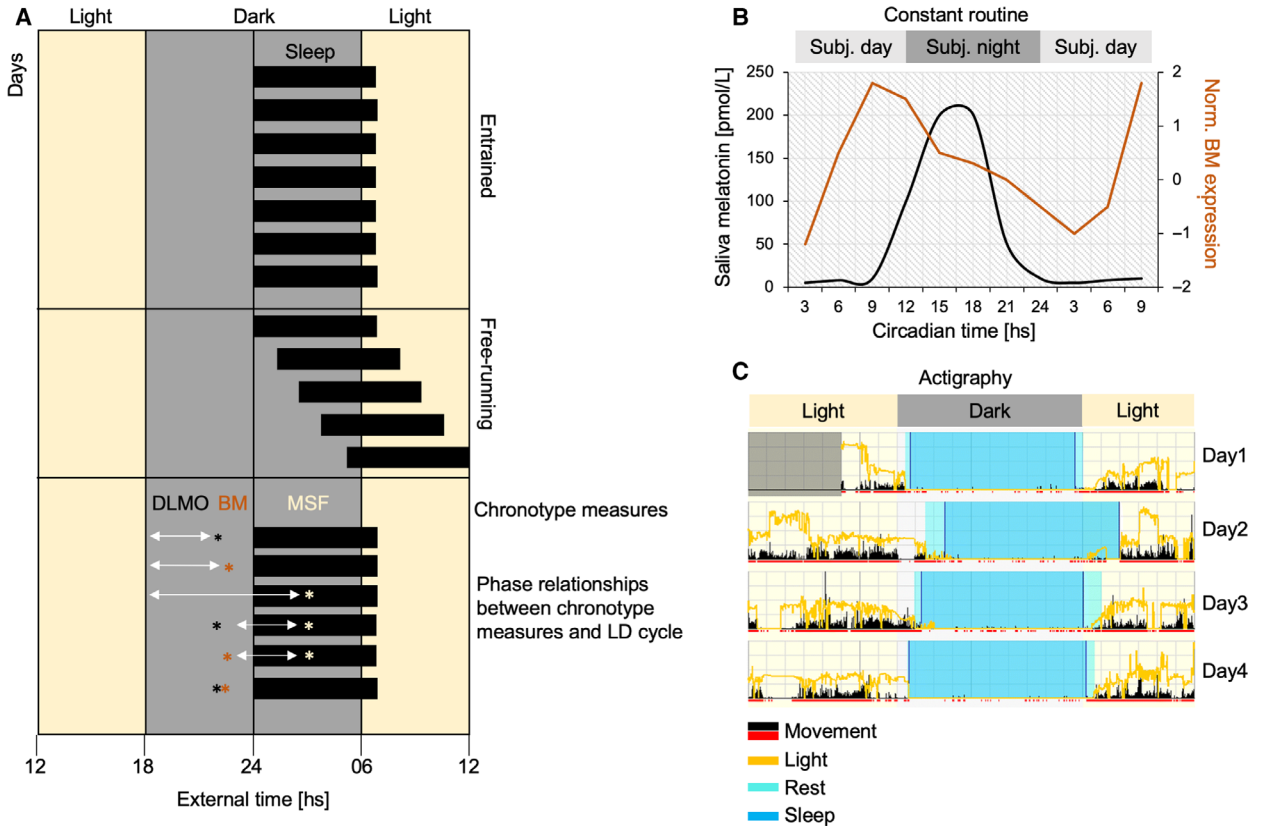


**Fig. 2.** Studying circadian rhythms in humans. (A) Schematic representation of human sleep–wake cycles under entrained and non-entrained conditions. Distinct phase markers for the assessment of endogenous circadian rhythms (DLMO, dim light melatonin onset; BM, biomarker; MSF, mid-sleep on free days) and their relative relationships to each other, as well as the light–dark cycle, are displayed. (B) Schematic representation of the oscillatory behavior of endogenous circadian clock markers measured under constant routine protocols (saliva melatonin and arbitrary biomarkers such as clock gene expression in blood). (C) Exemplary representation of actigraphy-based assessment of human sleep–wake cycles. Actigraph adapted from Ref. [443].

tissues, it is still not clear whether circadian clock parameters correspond to those of the central pacemaker, or whether they might be influenced by other Zeitgeber cues (intrinsic or extrinsic).

### Rest–activity and sleep–wake cycles

Measurements of human rest–activity and sleep–wake cycles can be performed over long durations and without large costs/effort by equipping subjects with so-called Actiwatches (wrist actigraphs), which record movement at 1- to 2-min intervals and sometimes also light exposure over several days (Fig. 2) [313,314]. However, even though rest–activity cycles often correlate well with melatonin and body temperature rhythms, they do not necessarily reflect the underlying circadian clock system as they may be affected by masking effects. Therefore, actimetry may be more useful for assessing entrainment state under varying exogenous or health conditions rather than an individual's circadian rhythm *per se* (for review, see Ref. [315]). In addition to actimetry, sleep–wake cycles can be assessed by questionnaires, sleep logs, sleep electroencephalography (EEG), or even more accurately by polysomnography (PSG). Especially for patients suffering from circadian rhythm sleep disorders, which are often caused by disruption of the endogenous clock system or misalignment between intrinsic and extrinsic rhythms, sleep assessment in addition to actimetry may be beneficial.

### Amplitude

Amplitude of circadian rhythms is an important characteristic as it can impact the entrainment range, phase of entrainment (chronotype), and PRCs to Zeitgeber stimuli. *In vivo*, amplitudes are difficult to determine since they are easily altered by a number of

confounding effects, for example, feeding–fasting cycles, activity, posture, or sleep. Studies have shown that amplitudes of melatonin, cortisol, or body temperature display interindividual differences and are attenuated with age [316–319]. However, whether these differences in amplitudes are governed by changes of the endogenous oscillator, whether and how they impact rhythmic biological processes, and how amplitude changes may arise remain to be investigated.

## Chronoepidemiology

Early epidemiological studies in chronobiology were interested in the distribution of human chronotypes across populations and how chronotype depends on, for example, gender and age. According to Roenneberg *et al.* [294], chronotypes, across large cohorts of German, Swiss, Dutch, and Austrian participants, are nearly normally distributed with a slight skewness toward later chronotypes. Chronotypes are both age- and gender-dependent, with chronotypes getting progressively later until the age of 20 before getting earlier again, and with males showing on average later chronotypes than females [294,320–323].

Modern lifestyle poses many challenges for the human circadian system and complicates synchronization of the endogenous circadian with exogenous Zeitgeber cycles. For example, artificial lightening and continuous food excess reduce the Zeitgeber strength of natural light–dark and feeding–fasting cycles. Travel across time zones (jetlag), social responsibilities (social jetlag), or shift work can lead to desynchronization of internal and external time, as well as among body clocks. Therefore, contemporary 'chronoepidemiology' often aims at identifying risk factors for the development of diseases associated with or arising from circadian misalignment.

With respect to chronotype, the discrepancy between work or school schedules and the endogenous timing system constitutes a problem that humans are facing on a daily basis. Chronotype distributions display a trend toward late types, especially during adolescence, yet work and school schedules are constructed for a population of morning types, promoting disruption of the endogenous circadian system. Evening types are more likely to suffer from social jetlag, that is, a substantial mismatch between sleep timing on workdays versus free days resulting from social obligations ([324]; for review, see Ref. [325]). Social jetlag promotes circadian misalignment and has been associated with reduced academic performance [326], obesity [327,328], unhealthy lifestyle [324,327,329], and changes in dietary patterns with increased caloric consumption during night shifts [330–332], decreased sleep and health-related life quality [333], depression [334,335], as well as metabolic diseases including T2D [336–339]. Noteworthy, the effects of chronic circadian misalignment on metabolic health are sex-dependent [340].

Recently, a large cohort study with UK Biobank volunteers identified associations between late chronotype and psychological, neurological, gastrointestinal/abdominal, and respiratory disorders, T2D, as well as with slightly increased mortality [341]. Notably, it has been reported that an about 1-h delay in school start time promotes longer sleep associated with higher grades, increased well-being, reduced sleepiness, and improved class attendance in teenagers [342,343], indicating that effects of social jetlag may be attenuated by adjusting daily life to the endogenous circadian system. In addition to late chronotypes, morning or intermediate types may be subjected to social jetlag and circadian disruption under shift work schedules. Due to light exposure at night, as well as disturbance of feeding–fasting and sleep–wake cycles, shift work promotes misalignment between external Zeitgeber and endogenous circadian rhythms or among body clocks, leading to adverse health consequences [344–346]. Not surprisingly, shift work has been associated with sleep, metabolic, and mental disorders, impaired alertness and cognitive functions, or even death [347–352] (for review, see Refs [353,354]). Recently, Hulsegge *et al.* [355] reported that morning types, as well as elderly workers (who tend to be earlier chronotypes), are more prone to suffer from shift work-induced sleep disturbances. In 2018, Stone *et al.* [356] reported that circadian PRCs display substantial interindividual difference in shift work-induced phase responses, which can be explained by differences in the amount of nightly light exposure relative to individuals' circadian phases. Moreover, circadian misalignment has been associated with non-24-h sleep–wake disorder (N24SWD) attributed to the lack of synchronization of the SCN to external Zeitgebers. N24SWD is mostly observed in blind people, although it was also reported in sighted individuals (reviewed in Ref. [354]). Thus, identifying individual differences in shift work tolerance, for example, associated with chronotype or photosensitivity, may be beneficial for avoiding health consequences from shift work-induced circadian disruption (for review, see Ref. [357]).

A topic of current interest in the field of chronoepidemiology is the impact of daylight saving time (DST) on the human circadian system. In 2018, the European Commission, based on an EU-wide online poll, decided to abandon DST and standard time switching

in EU member states, likely by 2021. DST during the summer months advances social clocks by 1 h, while the 'sun clock' remains the same. Thereby, work and school schedules are shifted earlier relative to sunrise and sunset, again promoting social jetlag, especially in late chronotypes. Moreover, prolonged exposure to high-intensity natural light in the evening may substantially delay body clocks and increase social jetlag in morning types (for review, see Refs [358,359]). Acute effects of the DST switch have been associated with reduced sleep duration and increased sleepiness, as well as with higher numbers of accidents, myocardial infarctions, ischemic strokes, and emergency room visits [360–364]. Chronic effects are more difficult to quantify. However, studies show that social jetlag is worsened during DST [365], suggesting that health consequences resemble those associated with social jetlag (see above). Additionally, previous attempts of introducing perennial DST in the United States and the United Kingdom have been abandoned due to their large unpopularity [366–368]. Whether the EU wants to introduce perennial DST or standard time, or whether all member states have to adhere to the same time standard is still under debate. However, in order to minimize effects of social jetlag, it is advisable to abandon DST and reassign regions to their natural clock times (based on sunset and sunrise) (for review, see Ref. [358]).

## The circadian clock system and human diseases

### Molecular clocks drive human physiology

Chronoepidemiological studies summarized in Approaches for studying human circadian clocks pinpoint that nearly all the aspects of human physiology and behavior are subject to temporal coordination by the circadian system. Similar to rodents (Peripheral clocks control cell and organ physiology: lessons from rodent studies), also in human individuals there is accumulating evidence that metabolic, cardiovascular, endocrine, digestive, and immune functions follow diurnal rhythms (Fig. 3) [47,222,224,225,227,228,281,369–373,375]. Although molecular clock studies in humans stay a challenging endeavor, analyses conducted in human saliva, serum, and urine serial samples obtained across 24 h revealed that large number of metabolites exhibits circadian rhythmic profiles [143,221,376–378]. Such rhythmicity is predominantly driven by the rest–activity cycle and not by SCN [379], and it is strongly affected by sleep deprivation [380,381], meal timing, and food type [382]. Similarly, temporal proteomics analyses conducted in human plasma identified strongly oscillating proteins, whose oscillations were blunted when participants had been subjected to acute circadian misalignment protocol [383]. Noteworthy, proteins involved in metabolic regulation comprising glucose metabolism were affected [383], highlighting the tight link between the circadian clock system and metabolism in physiological conditions, as well as in the development of metabolic diseases under perturbed clock conditions (discussed in details below and illustrated in Fig. 3). Recent studies conducted on serial ventral subcutaneous white adipose tissue (SAT) samples collected across 24 h under controlled conditions revealed that above 8% of the genes in ventral SAT of healthy lean individuals are circadian rhythmic, with the key regulators of metabolism, in particularly of the lipid metabolism, representing a major group among these genes [384,385]. Similarly, transcriptomic studies of human skeletal muscle biopsies collected from healthy volunteers revealed that genes involved in glucose and lipid metabolism exhibited strongly rhythmic temporal profiles [386]. Concordantly, lipidomics analyses demonstrated that above 20% of the lipid species including phospholipids, sphingolipids, and diacylglycerols were circadian rhythmic [387,388]. Moreover, lipid droplet size and content showed diurnal rhythmicity in type 1 muscle fibers [388]. While highly informative and allowing to unravel important insights into the human rhythmic physiology, these approaches are also highly invasive for the participant due to serial tissue biopsies, have limited time resolution, and are not applicable to most human tissues.

A highly instrumental and perhaps unique approach for dissecting human molecular clocks employing *in vitro* cultured human skin fibroblasts has been pioneered by Brown *et al*. [286,389]. Indeed, characteristics of oscillators assessed *in vitro* in primary fibroblasts derived from human skin biopsies and transduced with circadian bioluminescence reporters provided a very close estimation of the circadian system *in vivo* [286,389]. Noteworthy, disruption of circadian clocks by aging-related processes was mirrored in cultured fibroblasts assayed in the presence of serum from aged individuals [390]. Application of this powerful methodology to primary cells established from various human tissues paved the way to dissection of the molecular makeup of human clocks in different organs [312]. Thus, cell-autonomous circadian oscillations have been characterized in intact human pancreatic islets and in dispersed islet cells [391], human primary skeletal myotubes [392], white adipocytes [393], and primary thyrocytes [394].

Furthermore, studies of cultured primary tissue explants or primary cells synchronized *in vitro* allowed

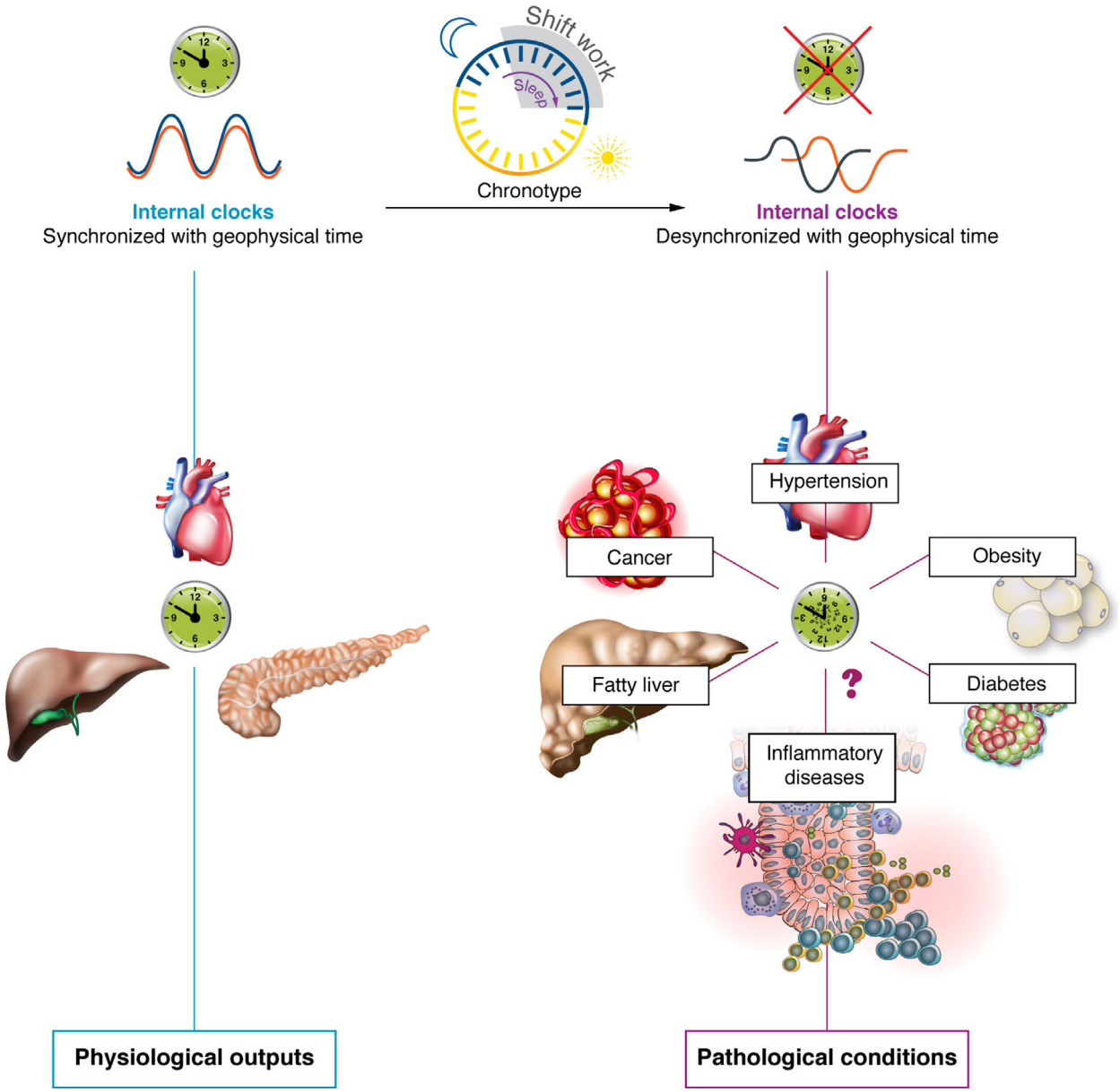


**Fig. 3.** When the clockwork goes wrong: human pathologies associated with circadian misalignments. The circadian timing system temporally orchestrates numerous aspects of body physiology and metabolism (left schema). When sleep or food intake occurs in desynchrony with the internal circadian time of the organism, a condition called 'circadian misalignment', promoting metabolic disorders comprising obesity, fatty liver disease, diabetes, cardiovascular diseases, hypertension, and cancer, can develop (right schema). Adapted from Ref. [224] with permission.

to gain significant insights into the transcriptional and functional outputs of the cell-autonomous clocks operative in human tissues [395]. In human skeletal myotubes, functional circadian oscillators were shown to drive gene expression of key metabolic genes, lipid homeostasis, myokine secretion, and glucose uptake [386,387,392]. Moreover, comparison of datasets stemming from experiments in primary cultured skeletal myotubes synchronized *in vitro* to skeletal muscle biopsies collected around the clock from healthy volunteers *in vivo* further supported the concept that cultured cells keep their transcriptional and metabolic landscape to a large extent [386,387]. Studies conducted in human pancreatic islets indicated that the circadian clock controls the expression of genes involved in the transport and secretion of insulin

[233,391,396,397]. Concordantly, circadian secretion profiles of insulin and glucagon by synchronized human islet cells were recorded *in vitro* [218,233,396,398]. The observed rhythmicity of insulin secretion was disrupted upon *CLOCK* knockdown in pancreatic islet cells [396], indicating that functional circadian clocks are crucial for regulating pancreatic endocrine function. Similarly, the expression of genes encoding key regulators of insulin signaling and glucose uptake was perturbed in human CLOCK-KO primary myotubes, concomitant with diminished glucose uptake by the muscle cells [386]. Taken together, these results consolidate the importance of the circadian system for normal glucose homeostasis and other essential metabolic functions, as well as its potential involvement in pathologies resulting from disruption of these processes.

## Circadian clock perturbation and human diseases

Growing evidence suggests that various human pathologies are associated with circadian misalignment between the internal clock system and external cues (Fig. 3; reviewed in Ref. [281]). Alterations in the circadian clockwork or in individual core clock components have been observed concomitantly with the development of cardiovascular, metabolic, immune, inflammatory, and mental diseases (Fig. 3) [228,370,384,385,394,399–402]. Genetic and molecular studies suggest that alterations in core clock components are associated with depression, bipolar disease, mood disorders, and intellectual disability [312,403–405], as well as that chronic circadian misalignment may lead to reduced cognitive performance [406]. Additionally, clock disruption has been associated with cancer progression [217,407]. For example, studies in human primary thyrocytes indicated that progression of papillary thyroid carcinoma is paralleled with altered synchronization properties of these cells [394]. Although causality often remains unexplored in these studies, changed expression levels of individual core clock genes have clearly been linked to the progression of oncogenic transformation, making them plausible candidates for diagnostic biomarkers [228,312,394,400,408–411].

Tight reciprocal connection between circadian system and metabolic cycles ensures proper temporal adaptation of metabolism to rest–activity and feeding–fasting cycles. Concordantly, perturbations of the clock system, due to aging or chronic misalignment, are associated with disruption of metabolic regulation and lead to the development of obesity and T2D (Fig. 3; reviewed in Ref. [47,223]). Circadian systems regulate resting energy expenditure and metabolism [412], and even short-term circadian misalignment has been demonstrated to promote reduced glucose tolerance by lowering insulin sensitivity [413]. The endocannabinoid system regulates hedonic eating that plays an important role in the etiology of obesity. Whereas temporal profiles of the endocannabinoid 2-arachidonoylglycerol (2-AG) in the blood of lean subjects exhibit pronounced circadian rhythmicity, it is significantly dampened and delayed in obese individuals [414]. In physiological conditions, circadian clocks drive diurnal rhythmicity of the glucose-regulating hormones insulin, glucagon, and GLP1 [218,222]. Strikingly, human pancreatic islets obtained from T2D donors displayed compromised molecular oscillations [398], as demonstrated by single islet and single-cell recordings, suggesting that the amplitude of islet cells is flattened and synchronization capacity compromised in T2D. Along with compromised cell-autonomous islet clocks, temporal profiles of insulin and glucagon secretion that display circadian rhythms in islets derived from non-diabetic donors were perturbed in synchronized T2D islets. Since in the model of clock disruption, insulin and glucagon granule docking and exocytosis have been found to be severely perturbed [398], it appears plausible that functional pancreatic islet oscillators influence islet hormone secretion *via* exocytosis process. Concordantly, the clock amplitude enhancing small molecule nobiletin [415] has been shown to boost the amplitude of circadian oscillations in T2D islets and to partly restore insulin secretory capacity of these islets [398]. Additionally, Nobiletin has been demonstrated to strongly counteract metabolic disorders in rodent models of obesity by enhancing clock protein levels [415], further strengthening the assumption that robust circadian rhythmicity is crucial for normal metabolic functions and prevention of metabolic diseases.

Glucose uptake by organs is rhythmic in healthy individuals [251,386,402]. By contrast, studies in T2D individuals report disruption of insulin sensitivity [384,402,416,417], alterations in the regulatory gene expression for glucose uptake in ventral SAT [384,385], and perturbed rhythm of core clock gene expression in leukocytes [418]. Consolidating the role of circadian misalignment in development of metabolic disease, chronic sleep deprivation and simulated shifted work in healthy subjects have been shown to cause reduced glucose tolerance [419–421] and insulin resistance [402,422,423]. Moreover, induced circadian misalignment resulted in perturbed glucose tolerance and metabolite rhythms [402,424–427]. Noteworthy, and in line with studies based on the laboratory

protocols of sleep deprivation and shifted activity, observational studies in shift workers reported significantly increased risk of developing T2D that correlated with the number of night shifts per month [428]. Interestingly, whereas no differences in the clockwork of primary skin fibroblasts and skeletal myotubes were detected between groups of non-diabetic lean, obese, and T2D individuals [399,429], *BMAL1* oscillation period measured in primary skin fibroblasts has been reported to be inversely correlated with HbA1c values in the blood of individuals from the T2D group. This conjunction further speaks for the interconnected relationship between T2D progression and the properties of individual core clock components and highlights the potential of the clockwork assessment not only for disease diagnostics, but also for assessment of disease progression and clinical severity [399]. In line with these findings, transcriptional analyses of T2D human islets have been reported to altered expression of *PER2/3* and *CRY2* [430,431].

## Perspectives

Today, increasing numbers of immediate clinical applications emerge from recent studies of human clocks in their physiological state, as well as under pathological conditions. Utilizing individual clock properties as molecular biomarkers holds promise for personalized medicine approaches, as well as for diagnostic purposes [372,408,432]. Chronopharmacology that takes into account circadian pharmacokinetics and pharmacodynamics already plays an important role for a number of medications widely used for treating oncological, metabolic, and respiratory diseases [433,434]. Additionally, the rapidly developing field of chrononutrition emphasizes the importance of meal timing for prevention and treatment of metabolic diseases [432,435–437] (for review, see Ref. [438]). In addition, timely scheduled exercise holds promise for the restoring misaligned circadian clocks [439]. Lastly, exploring the roles of the circadian clocks in host–pathogen interactions, especially in view of unfolding epidemics [281,440,441], as well as functions of small-molecule clock modulators [271,398,442], bears significant potential for the development of therapeutic strategies targeting diseases associated with circadian disruption and misalignment.

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