

## Synchronization of the mammalian circadian timing system: Light can control peripheral clocks independently of the SCN clock

Alternate routes of entrainment optimize the alignment of the body's circadian clock network with external time

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A vast network of cellular circadian clocks regulates 24-hour rhythms of behavior and physiology in mammals. Complex environments are characterized by multiple, and often conflicting time signals demanding flexible mechanisms of adaptation of endogenous rhythms to external time. Traditionally this process of circadian entrainment has been conceptualized in a hierarchical scheme with a light-reset master pacemaker residing in the hypothalamus that subsequently aligns subordinate peripheral clocks with each other and with external time. Here we review new experiments using conditional mouse genetics suggesting that resetting of the circadian system occurs in a more federated and tissue-specific fashion, which allows for increased noise resistance and plasticity of circadian timekeeping under natural conditions.

### Keywords:

circadian clock; clock genes; entrainment; light; mammals; peripheral clocks; SCN

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### Introduction

The circadian system provides a first-rate example illustrating in what way changes in the environment shape behavior and physiology. The 24-hour periodicity of axial rotation of our planet has favored the evolution of circadian clocks, endogenous cellular *pacemakers* (terms in italics throughout the text are defined in Box 1) that allow organisms to adapt to the natural light-dark rhythm. Circadian clocks are present in unicellular organisms such as dinoflagellates [1], in plants [2, 3], in insects [4], and in vertebrates [5]. The first gene encoding a critical component of a circadian clock (*Period*) was discovered in *Drosophila* by Konopka and Benzer in 1971, showing that circadian clocks are genetically encoded [6]. In mammals, circadian clocks are found in nearly all cells and tissues. At the biochemical level, they consist of coupled feedback loops that collectively establish a self-sustained, yet adjustable molecular oscillator that controls, via transcriptional programs, a wide spectrum of cellular and organismal processes (Fig. 1). A critical feature of circadian clocks is their ability to sustain circadian oscillations for days or even weeks, even in the absence of an external periodic stimulus. This property is maintained even in tissue explants and cell cultures [7, 8].

### Organization of the circadian system

Because of the widespread presence of clocks throughout the body, the circadian system of an animal resembles a clock shop rather than a single clock. Therefore, the important question arises of how rhythms of so many clocks are efficiently synchronized. There are two obvious ways of

**Box 1****Abbreviations and terminology**

**DD:** Constant darkness; used to analyze free-running rhythms of the circadian clock.

**Entrainment:** The coordination of a self-sustained oscillator via rhythmic signals from a pacemaking oscillator (i.e. a zeitgeber). In a loose way, this refers to the process of alignment of the internal clock with external time.

**Free-run:** Behavior of internal clocks under Zeitgeber-free conditions. When free-running, circadian rhythms cycle with their endogenous period which usually deviates from 24 hours. For example, in the C57BL/6 mouse strain, the free-running period in DD is roughly 23.7 hours. However, this value differs with strain, species, and light intensity.

**Jetlag:** Misalignment of internal and external time after rapid crossing of several time zones. Re-entrainment of circadian clocks to local time ends jetlag after a number of days depending on the magnitude of the time shift.

**LD:** Rhythmic light:dark conditions. While the time intervals can differ depending on experimental setup, in this paper, we refer to standard 12 hours light:12 hours dark conditions, which synchronize central and peripheral clocks when no conflicting Zeitgeber signals are present.

**Masking:** Masking refers to a direct regulation of an overt rhythm while over-riding entrainment, or enforcement of

periodicity independent of circadian control. Masking does not affect clock function and does also not depend on a functional circadian system.

**Pacemaker:** A central clock (i.e. the SCN) that coordinates rhythmic output of peripheral clocks and aligns them to external time.

**Peripheral clocks:** Cellular oscillators outside the SCN; found in peripheral tissues, but also in various brain areas. An alternative designation is subordinate clocks, emphasizing their dependence on SCN input.

**Phase angle:** Refers to the temporal position of a landmark point of an expressed internal rhythm with respect to an external reference, e.g. the time of sleep onset relative to the time of “lights off.”

**RF:** Time-restricted feeding; a potent zeitgeber for peripheral tissue clocks.

**SCN:** Suprachiasmatic nucleus, a hypothalamic nucleus above the optic chiasm considered to be the circadian pacemaker in mammals.

**TTL:** Transcriptional-translational feedback loop; the core organizational unit of the circadian clock (see Fig. 1A).

**VIP:** Vasointestinal polypeptide; peptide mediator of the hypothalamus and the gut.

**Zeitgeber:** A rhythmic signal capable of entraining circadian clocks.

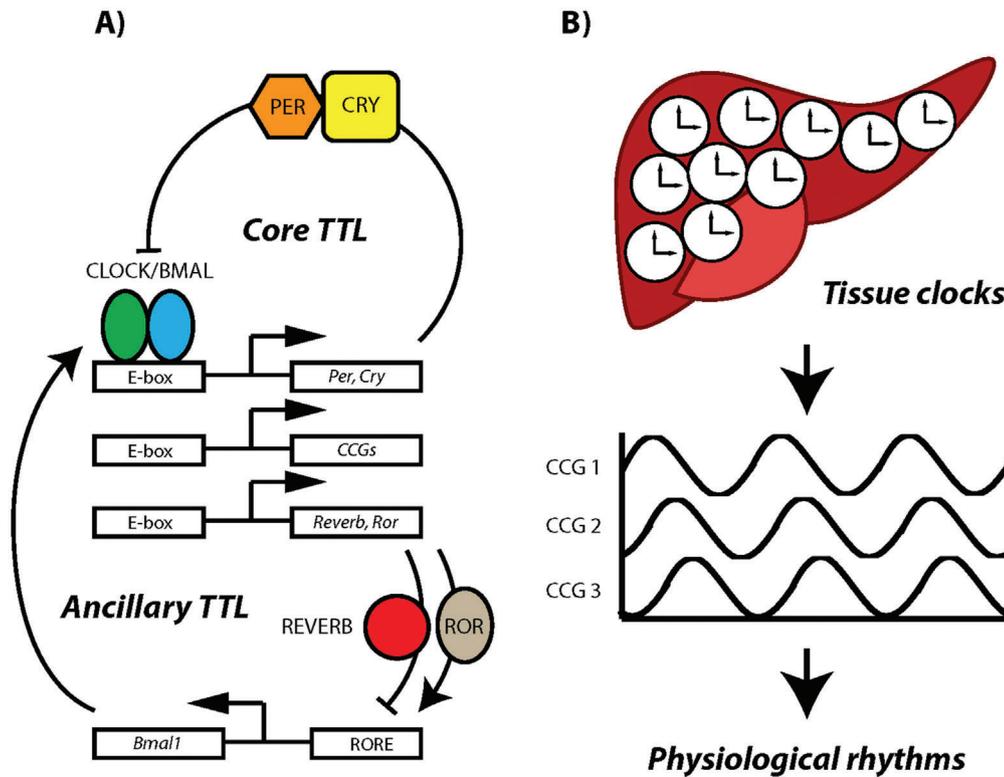
circadian rhythm coordination. One assumes a master pacemaker that instructs all subordinate, i.e. peripheral, clocks (hierarchical system). The other mechanism would depend on coupling of a multitude of essentially equivalent oscillators (“federated” system). In mammals, the discovery of a central circadian pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus long suggested a strict top-down control [9, 10]. Because the SCN receives direct input from the retina via the retino-hypothalamic tract, this nucleus serves as a direct window looking into the outside world. The neurons within the SCN are coupled and as an ensemble produce electrical and molecular circadian rhythms of a robustness not seen in isolated neurons and other types of cells [11]. The SCN is connected to several other hypothalamic and extra-hypothalamic nuclei thereby becoming part of an intricate neuronal network [12]. A vast body of literature supports a hierarchical control of circadian synchronization, in which the rhythm of the master pacemaker in the SCN is *entrained* by an external *zeitgeber*: the rhythmic change of light and dark. The SCN subsequently synchronizes *peripheral clocks* with each other and thus aligns the entire circadian system to the external light-dark cycle. The detailed nature of the synchronizing signals is still being investigated, but relies on a combination of humoral factors and the peripheral autonomic nervous system (ANS) [16, 17].

This model of a strictly SCN-centric circadian control has been challenged. Timing signals other than light, such as rhythmically timed food intake, can potentially reset peripheral clocks directly without affecting clock rhythms in the SCN [13, 14]. Ishida and co-workers showed that light exposure can acutely activate clock gene expression and glucocorticoid

release in the adrenal gland via sympathetic innervation by the splanchnic nerve [15], and this effect appears to be independent of photic responses at the level of the SCN [16]. A more “federated” organization of independent oscillators offers the opportunity of different zeitgeber signals acting independently on different peripheral clocks. For example, timed food uptake could primarily reset the liver clock and thereby regulate liver metabolism. The light-dark cycle could primarily entrain the SCN and its output such as rhythmic locomotor activity [17]. The challenge for the “federated” control will be to identify the signaling routes that ensure that all the clocks of the clock shop are properly coordinated.

It should be recalled that under natural conditions, many zeitgeber signals are tied to the day-night cycle (Fig. 2). This potent photic zeitgeber evokes (in most species) a 24-hour sleep-wake rhythm. Sleep precludes both food intake and locomotor activity. Thus, the sleep-wake rhythm effectively, yet indirectly, drives food intake and body temperature cycles. Nonetheless, one can uncouple light and food: when sleep rhythms are disrupted (e.g. in the case of *jetlag*) or food intake is restricted to the natural sleeping *phase*, alignment of different zeitgeber signals is impaired, leading to an uncoupling of SCN and peripheral clocks [14, 18]. Many insights into the circadian mechanisms stem from such experimental interference studies.

Excellent reviews on the molecular mechanism of circadian timekeeping have recently appeared [5, 19–22], some of which also address the clock coordination problem (e.g. [20]). In this paper, we discuss to which extent the circadian clock of the SCN is required for peripheral clock synchronization with an emphasis on experimental strategies



**Figure 1.** The circadian molecular clockwork. **A:** At the molecular level circadian clocks are based on interlocked transcriptional/translational feedback loops (TTLs). In the circadian core TTL the transcription factors CLOCK and BMAL1 activate the expression of *Period* (*Per1–3*) and *Cryptochrome* (*Cry1/2*) genes during the day. PER and CRY proteins accumulate in the cytoplasm and toward the night translocate to the nucleus where they inhibit CLOCK/BMAL1-mediated transcription. Accompanied by modifications of histone tails around the DNA-binding sites of CLOCK/BMAL1, PER/CRY binding leads to a dissociation of the transcription complex and suppresses production of *Per* and *Cry* mRNAs. Gradual degradation of PER/CRY complexes toward the end of the night releases the CLOCK/BMAL1 dimer from PER/CRY suppression, thus re-initiating the clock cycle by induction of *Per* and *Cry* transcription. A number of ancillary feedback loops [73–75] as well as post-translational mechanisms [76] fine-tune and stabilize the oscillation of this core TTL. In addition to creating a self-sustaining clockwork, core clock proteins also control transcription of a large number of tissue-specific clock-controlled genes (CCGs). **B:** Typically each cell of an organ such as the liver houses its own clock which drives the circadian expression of CCGs to create organ-specific rhythmic physiological responses. Although the clock components may differ between species, this principle organization of circadian timekeeping is preserved in all animals and plants.

in which the SCN clock was genetically perturbed, an approach complementary to classical SCN lesion and transplantation. In addition, we review work in which the SCN was ablated and the response of peripheral clocks was investigated by non-invasive analyses. We discuss these data in relation to the hierarchical and “federated” models. It is worth noting that recent work in *Drosophila* suggests a revision of a hitherto widely accepted dual-oscillator model in which pigment-dispersing factor (PDF) positive neurons were thought to serve as master pacemakers controlling PDF-negative output neurons. The new findings suggest that rhythms result from an interaction between multiple independent oscillators [23].

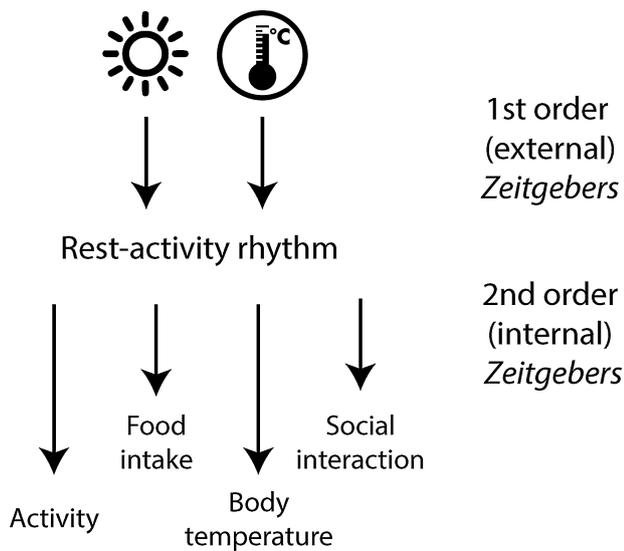
neuronal networks. Since the SCN is also part of such a network, it is necessary that classical SCN surgical experiments are complemented by alternative approaches [33] such as SCN-specific deletion of clock genes that preserves the neuronal circuit.

#### Light can synchronize peripheral clocks in mice with *Syt10*-driven deletion of *Bmal1* in the SCN

Husse and colleagues recently developed a *Syt10*-driven CRE line to delete – in a floxed *Bmal1* mouse [34] – the essential clock gene *Bmal1* predominantly in neurons of the SCN while

#### New genetic tools enable functional manipulation of the SCN circadian clock

Complete bilateral lesions of the SCN evoke arrhythmicity of locomotor activity, drinking behavior, hormone release, and body temperature [9, 10, 24]. Tissues from SCN-lesioned animals show abolished clock gene rhythmicity under both LD and DD conditions [25–30]. These lesion experiments, in combination with SCN transplantation studies [31, 32], thus show that the SCN controls coherent behavioral and molecular rhythmicity in intact organisms. A significant limitation of lesions is that they not only ablate the structure in question, but also interrupt the associated



**Figure 2.** Different zeitgeber signals synchronize the circadian clock. As the endogenous period of the circadian clock is only approximately 24 hours, it has to be entrained to the external 24-hour cycle every day. Zeitgeber signals are stimuli that can reset the circadian clock. The major external zeitgeber signals (first order signals) are the light-dark cycle and external temperature changes. In addition, internal zeitgeber signals (second order signals) ensure the synchronization of different body clocks with each other and with external time. The most important internal zeitgeber signals are locomotor activity, the timing of food intake, body temperature, and social interaction.

leaving *Bmal1*, and hence clocks, unaffected in peripheral tissues [35]. BMAL1 immunohistochemistry, in situ hybridization of clock and clock output genes, as well as PER2::LUC explant recordings from control and mutant SCN all showed that experimental parameters were near base level in mutant SCN supporting the notion that the SCN clock was strongly impaired [36]. In line with this, mutant mice did not exhibit circadian rhythms of locomotor activity in DD conditions [35]. In LD conditions, however, 24-hour activity rhythms were preserved, indicating that photic *masking* was by and large maintained – which is not the case in SCN-lesioned mice. After SCN lesioning peripheral clock rhythms are abolished. In mice in which solely the SCN clock function was impaired high-amplitude rhythms of clock gene expression were maintained, and rhythms were synchronized to the external light-dark cycle and with each other [36]. This indicates that SCN clock function is dispensable for the synchronization of peripheral clocks under these conditions. Circadian profiling of clock gene expression and PER2::LUC explant rhythms revealed that peripheral clocks initially were capable of sustaining synchronized rhythmicity under zeitgeber-free conditions in the absence of the SCN pacemaker. However, when PER2::LUC rhythms were again assessed after 7 days, overt rhythms were lost due to desynchronization between free-running tissue oscillators. Husse et al. monitored fecal glucocorticoid levels, a robust endocrine clock marker that can be tracked non-invasively over several days. In LD, glucocorticoid levels were strongly circadian, but upon release into DD a gradual dampening of rhythms was observed resulting in a complete

loss of circadian rhythmicity after 7 days in the zeitgeber-free environment. When the circadian system was challenged by subjecting SCN clock-deficient mice to an abrupt shift in the LD cycle (jetlag paradigm), the absence of the SCN clock resulted in accelerated resetting of PER2::LUC rhythms in peripheral tissue explants. Such hastened light adaptation of peripheral clock rhythms in the absence of SCN pacemaker function suggests that, in a wild-type animal, the SCN clock stabilizes the phase of the peripheral circadian system against external perturbation. In summary, these findings suggest that under LD, but not DD, conditions the SCN clock is dispensable for the synchronization of peripheral clocks. Importantly, while putting into perspective the coordinating function of the SCN in circadian rhythm regulation, the mouse genetic findings immediately propose an important function for the central pacemaker: if the circadian system is challenged by an absence of zeitgeber information (DD) or when conflicting zeitgeber signals interfere (jetlag), the SCN clock becomes essential to steady the circadian cellular oscillator network.

It is debatable whether such alignment of peripheral clocks with the external light-dark cycle qualifies as entrainment in the classical sense (see [37] for a detailed discussion of entrainment theory), which requires the existence of two independent and self-sustained oscillators. The fact that peripheral clock gene rhythms are sustained at least for some time under DD conditions suggests that these can be seen as oscillators of limited self-sustainment, comparable to a pendulum that due to friction will eventually cease swinging, unless it is periodically deflected. One also has to keep in mind that the loss of circadian rhythmicity at the organ level does not necessarily imply that individual cells are arrhythmic but might also be due to a loss of intercellular synchrony.

### Light can synchronize peripheral clocks in mice with *CamK2*-driven deletion of *Bmal1* in the SCN

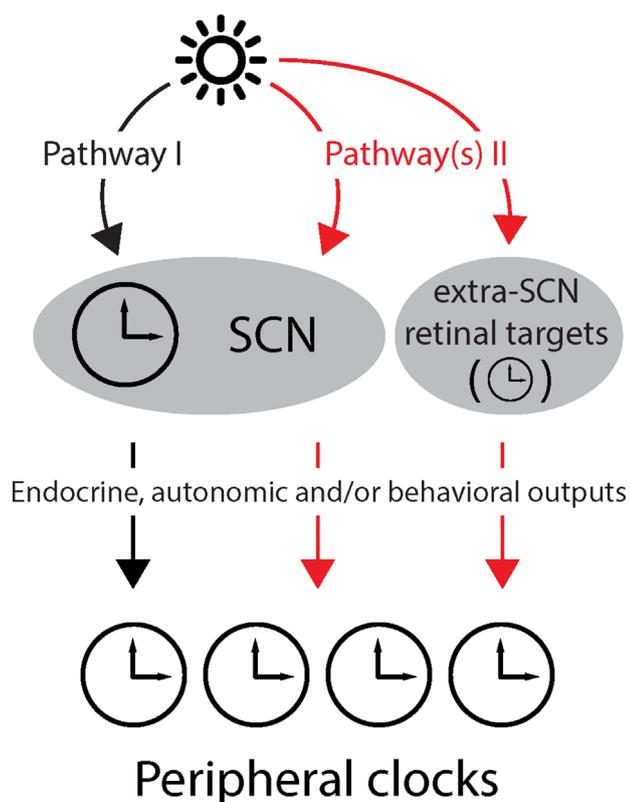
The findings of Husse et al. were buttressed by a study using a *CamK2*-CRE driver line to abolish *Bmal1* throughout the forebrain, including the SCN, while leaving peripheral tissues unaffected [38]. *Bmal1* deletion in the SCN was efficient, as shown by attenuated clock gene rhythms as well as a loss of circadian locomotor activity in DD. Mutant mice were rhythmic in LD; the locomotor activity onset, however, was more variable and appeared slightly phase-advanced when compared to controls. The authors also investigated the masking response in more detail and found an impairment of light-induced suppression of behavioral activity in mutant mice. Clock gene expression in peripheral tissues of mutants exhibited well-phased rhythms in LD conditions, indicating that internal synchrony was maintained. In DD, peripheral clock gene expression profiles showed dispersed phasing as well as reduced oscillatory amplitudes. Using high-resolution bioluminescence imaging of heart tissue, Izumo and co-workers demonstrated that the decreased amplitude of peripheral clocks was due to a combination of decreased cellular rhythmicity and increased phase desynchrony between cellular clocks [38]. In an additional set of experiments, *time-restricted feeding (RF)* in DD was used to rescue

peripheral clock regulation in forebrain clock-deficient mice. Feeding cues synchronized circadian clocks in liver and kidney, while peripheral clocks in heart, lung, and spleen were less responsive to timed food intake. The oscillatory amplitude was restored to a level comparable to control mice only in liver, while all other peripheral tissues showed lower amplitude oscillations even after 2 weeks of RF. The authors concluded that internal synchrony and high-amplitude peripheral rhythms vanish in the absence of a functional SCN pacemaker. However, rhythms can be restored by the external zeitgeber light or, at least partially, by timed feeding [38].

In summary, both studies conclude that despite a genetic ablation of the SCN clock peripheral clock rhythmicity and synchrony is largely maintained in the presence of an external zeitgeber. While light resets all tissues, feeding cues only partially restore internal synchrony in SCN clock-deficient mice. In the absence of any zeitgeber peripheral rhythms are attenuated and internal synchrony is markedly impaired. Together these findings provide strong genetic evidence for the notion that in addition to the sequential pathway, in which light synchronizes the SCN clock, and the SCN clock in turn transmits the timing information to peripheral clocks (Fig. 3,

pathway 1), there exist additional pathways by which peripheral clocks are synchronized independently of the SCN clock (Fig. 3, pathway(s) 2). At this point, the nature of these pathways remains to be explored. Light-driven activation of SCN neurons may lead to rhythmic autonomic, endocrine, or behavioral outputs that then translate into rhythmic clock gene activation in peripheral tissues. Alternatively, various hypothalamic and some extra-hypothalamic regions outside the SCN receive retinal input and might influence endocrine, autonomic, or behavioral outputs that regulate the periphery [39]. Some of these areas may even house endogenous, light-entrainable circadian clocks. SCN ablation studies seemingly have missed out on such additional pathways.

It is important to realize that despite the use of different CRE driver lines to delete *Bmal1* in overlapping, but not identical, regions of the brain (compare [42] and [37]), both studies gave very similar results. It is well known that CRE driver lines have limitations with regard to tissue specificity and recombination efficiency. Therefore, the use of two independent drivers targeting the same gene (*Bmal1*) strengthens the conclusions that can be drawn from this genetic approach.



**Figure 3.** Parallel pathways synchronize peripheral clocks to the LD cycle. Light is the most important zeitgeber for circadian clocks and can reach peripheral clocks via several routes. In pathway 1, light from the retina reaches the SCN through the retino-hypothalamic tract and entrains the SCN clock that then transmits the timing information to peripheral clocks along neuronal and/or endocrine pathways. Pathway 2 depicts additional routes for transmission of photic information that bypass the SCN clock and directly or indirectly reset peripheral clocks.

### In vivo PER2::LUC recordings in anesthetized mice reveal SCN-independent peripheral clock resetting

Recent advances were also made in the use of real-time, non-invasive analysis of the output of mammalian circadian clocks. High-resolution time series have long been available for locomotor activity [40] and for secretion of glucocorticoids [41, 42], but directly following molecular clocks inside the body was achieved just recently [43, 44].

Tahara et al. [44] used in vivo bioluminescence monitoring in *PER2::LUC* mice bred onto an albino ICR background. For measurements of luminescence animals were repeatedly anesthetized with isoflurane, and luciferin was infused to elicit light emission through luciferase-mediated oxidation. Kidneys, livers, and submandibular glands were monitored. In all three tissues *PER2::LUC* activity was rhythmic with peaks in the first half of the dark phase. In tissues of *Bmal1-LUC* mice, in which luciferase expression is driven by the *Bmal1* promoter, luminescence rhythms were shifted by 8 hours relative to those observed for *PER2::LUC*. Apparently, the reporters recapitulate the naturally occurring anti-phasic expression profiles of endogenous *PER2* and *Bmal1*. After SCN lesion, peripheral tissue rhythms were rhythmic but greatly dampened, and normal phase relationships between tissues were abolished. This contrasts with previous data obtained from real-time quantitative PCR (qPCR) experiments with SCN-lesioned animals where peripheral clock gene rhythms were completely lost [27]. Such discrepancy may be due to the fact, that qPCR profiles usually combine average data from different animals [27]. While these findings do not devalue the old studies, they emphasize the necessity of time series from individual animals to overcome inter-animal variations in gene activity levels.

To assess free-running periods of different tissue clocks, Tahara et al. adapted SCN-lesioned mice to RF under LD conditions and then subjected them to ad libitum food access and DD [44]. Under RF/LD conditions PER2::LUC rhythms in kidney, liver, and submandibular gland were reset by 12 hours compared to ad libitum food/LD consistent with what had been observed from sequential sampling studies before [14]. Under free-run conditions, rhythmicity in peripheral clock activity was maintained at a period of slightly less than 24 hour, reminiscent of what is observed for locomotor activity in wild-type mice. However, rhythm amplitudes became dampened soon after release into ad libitum food/DD, indicating that a functional SCN pacemaker coordinates and maintains peripheral rhythmicity in the absence of an external zeitgeber.

### PER2::LUC recordings in freely moving mice reveal accelerated resetting of the liver clock in SCN-lesioned mice

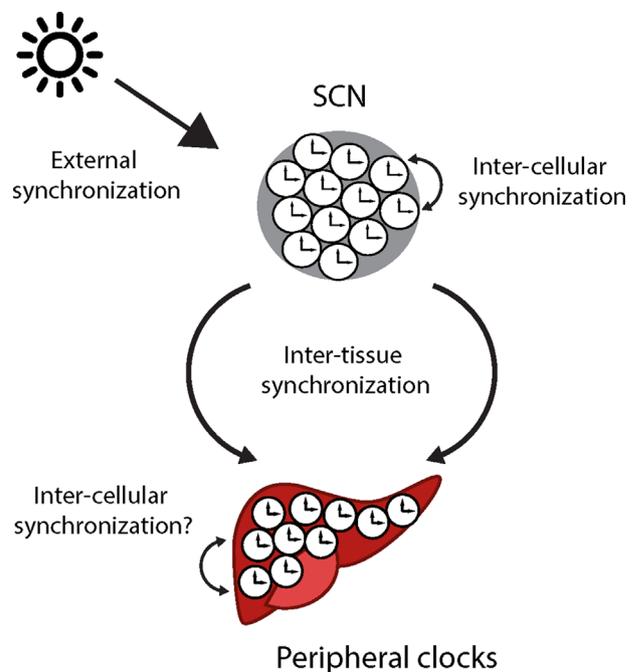
Saini et al. [43] have invented a device that houses an individual mouse that has access to food and water, whose locomotion can be monitored by infrared sensors and whose bioluminescence photons emitted (e.g. from liver) are collimated and recorded by highly sensitive photomultiplier technology. Bioluminescence in liver was evoked by transducing hepatocytes with an adenoviral *Bmal1-LUC* reporter introduced by tail vein injection. Luciferase substrate was delivered continuously with an osmotic mini pump implant. Saini and co-workers used this set-up to investigate the regulation of hepatocyte clocks by SCN and food entrainment signals [43]. Animals maintained under night-restricted feeding were shifted to daytime-only feeding, and the phase shifting of liver bioluminescence rhythms was recorded for several days at 1-minute intervals. Animals with an intact or a sham-operated SCN responded slowly to a shift in the feeding regimen. By contrast, mice with SCN lesions showed a rapid phase-shift in their hepatic BMAL1-LUC activity rhythms indicating that SCN function shields the liver clock from food-induced phase shifting.

As is often the case, new methods and technologies shed new light on long-standing questions. With regard to the essentiality of a master pacemaker in the SCN, the recent work suggests that a genetic deletion of *Bmal1* in the SCN only to some extent affects the amplitude and phase of peripheral clocks, as long as animals are maintained in an LD environment. The rhythmic change of light and dark is sufficient to impose a rhythm of clock gene expression in peripheral organs, even if the SCN clock is completely abolished. If such SCN-clock deficient mice are maintained in a zeitgeber-free (e.g. DD) environment, peripheral clock gene expression rhythms become phase-dispersed and their amplitude is strongly reduced, presumably because cellular synchronization in peripheral tissues is abolished. In mice with an intact SCN, neither timed feeding nor a reversal of feeding time has an immediate consequence on the activity rhythm of a hepatic luciferase reporter. In contrast, in mice in which the SCN was surgically ablated, timed feeding imposes

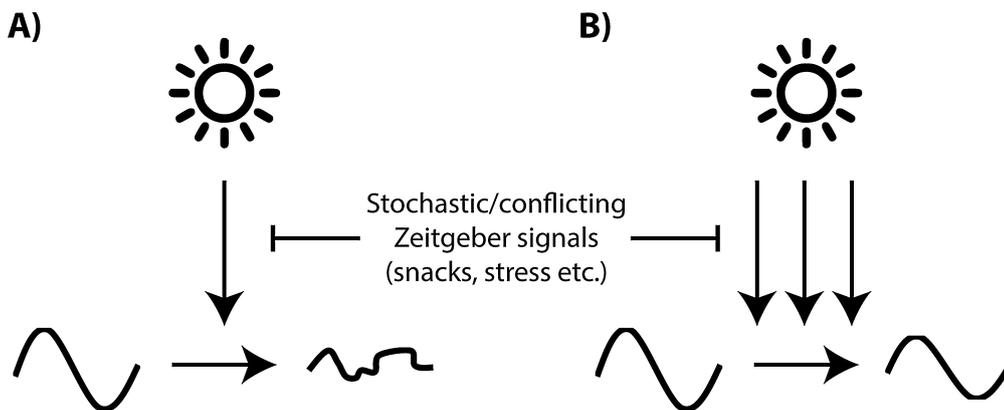
a rapid shift in hepatic luciferase reporter activity rhythms, thus revealing the existence of nutrient-dependent zeitgeber cues that substitute for the SCN signal. What is the biological relevance of such metabolic cues? Envisage a scenario where a nocturnal rodent has limited access to food at dusk. If metabolic enzymes required for the uptake and processing of nutrients were entirely subject to regulation by the SCN clock, the clock controlled enzymes required for digestion might not be optimally expressed. This problem is resolved, if food uptake itself serves as a zeitgeber to turn on the expression of clock controlled metabolic enzymes. Such decentralized control provides the organism with the opportunity to integrate complex periodic changes (light, food, temperature, humidity, social cues, etc.) that occur in the surroundings over the 24-hour period and over which the organism has no control.

### Organization of the “federated” multi-clock system

The studies reviewed above, favor a “federated” model of the circadian timing system. This raises a host of questions such as how the myriads of peripheral tissue clocks are synchronized with each other and with external time (Fig. 4).



**Figure 4.** Types of synchronization within the circadian timing system. Several synchronization mechanisms are required to ensure internal and external synchrony of the numerous cellular clocks present in higher organisms. Single cells within a tissue have to be synchronized with each other (inter-cellular organization) to allow synchronized circadian output of a particular peripheral tissue clock. In addition, different tissue clocks have to be synchronized with each other, to maintain systemic internal synchrony. Lastly, the entire organism has to be synchronized to the external 24-hour LD cycle to ensure temporal adaptation (external synchronization).



**Figure 5. A:** Redundant synchronization pathways protect the circadian system against zeitgeber noise. In a scenario where a single pathway resets the circadian clock, external perturbations such as conflicting zeitgeber signals (e.g. from occasional food intake during the rest phase) would potentially have marked effects on the amplitude and phase of the circadian system. **B:** Redundant synchronization pathways protect the clock network against such sporadic external zeitgeber signalling and allow high-amplitude and synchronized circadian clock rhythmicity even under complex (and noisy) environmental conditions.

Intercellular and inter-tissue synchronization is particularly challenging because most tissues contain multiple cell types. Little is known about the mechanisms of intercellular synchronization except for the SCN in which paracrine signals (*VIP* and others), gap junctions, and electrical signaling appear to play a role in keeping SCN cell rhythms aligned with each other [45–48]. In the case of peripheral clocks, mechanisms of intercellular synchronization are largely elusive. Some studies suggest that cellular coupling is confined to the SCN [11]. It should be recalled, however, that while in dispersed cell cultures overt rhythms are lost within 2–3 days, clocks in explants of peripheral tissues retain high-amplitude circadian oscillations for substantially longer [7]. These findings support the existence of short-range signaling through direct cell-to-cell communication, for example via gap junctions in endothelial tissues, or by paracrine factors. In development, such short-range interactions are mediated by transmembrane signaling based on direct interactions between cell surface receptors and their cognate ligands. Whether this signaling mechanism occurs in the circadian timing system remains to be seen. It is also unclear whether in a multicellular tissue all cellular clocks are equal. It is tempting to speculate that the clock in a specific cell type might set the phase of the clock in neighboring cells as was proposed for Clara cells in the lung epithelium [49].

Rhythmically released hormones such as melatonin or glucocorticoids, other blood-borne signals [50], or even body temperature cycles could, either alone or in combination, sustain synchronization of multiple peripheral clocks. The timing of each of these signals may also be controlled by the autonomic nervous system and perhaps by other tissue clocks, such as the adrenal [51]. Additional control of inter-tissue synchronization may stem from hypothalamic nuclei, many of which contribute to the regulation of physiological functions

known to be clock-controlled [52]. Synchronization to environmental zeitgebers are well-characterized in the case of photic entrainment of the SCN clock [53], but there are only a few studies on non-SCN tissues [15, 54].

### A “federated” clock system increases temporal stability and plasticity

From an evolutionary perspective, the question arises why it would be beneficial for multicellular organisms to maintain a complex “federated” network organization of clocks instead of a seemingly more straightforward hierarchical organization with a master regulator controlling coherent circadian physiology across the body. One possible explanation would be that cellular clocks took over additional adaptive functions such as coordinating tissue-specialization and cross-tissue coordination resulting in increased overall fitness. Additional rationales are increased resilience to conflicting zeitgeber signals due to parallel and largely independent routes of synchronization and increased plasticity in response to complex environments and, thus, environmental demands.

The multimodality of resetting mechanisms characterizing the “federated” clock model allows for a more differentiated network response. At the same time, by routing the time signal through different paths, it increases the overall stability of the system, protecting it against conflicting zeitgeber signals (Fig. 5). Supporting this notion, Husse et al. have shown that peripheral clock adaptation to a shifted LD cycle is accelerated if only one resetting pathway is functional [36]. Similarly, peripheral clocks in SCN-lesioned mice reset faster to a shifted feeding schedule [43]. Under artificial conditions such as jetlag increased internal phase stability becomes a liability, because one would want clocks to adapt fast. In a more natural and, thus, rather noisy zeitgeber environment, however, it proves highly advantageous, because it prevents the system from unwanted phase shifts in response to stochastic or intermittent zeitgeber signaling.

Another potential advantage of a “federated” circadian network is increased network plasticity in the context of complex environmental conditions. As described above, different clocks respond differently to certain timing signals. For example, the liver as a metabolic organ is highly sensitive to RF while the SCN controlling, e.g. locomotion is primarily

reset by light [14]. A strictly centralized network would result in synchronization of all physiological processes to the same zeitgeber signal. A “federated” network, however, allows for each clock and, therefore, each physiological process to be synchronized to those zeitgeber signals that are most relevant for a particular process, resulting in a tailored response. For example, the liver clock should be synchronized to rhythms in food intake, but it should also respond to changes in energy demands or variations in oxygen supply. A “federated” organization allows for better adaptation to changing environments. For example, seasonal adaptation of circadian rhythms might be facilitated by differential resetting of tissue clocks in response to changes in photoperiod, temperature, humidity, and other parameters that change over the course of a year. It has been suggested that the phase distribution of individual SCN cells contains information about day-length [55] and that coupling of individual SCN cells facilitates photoperiod adaptation [56].

### Future routes of research

It will be of interest to further explore the different synchronization pathways in SCN clock-deficient mice. Such experiments should focus on modifying the circadian rhythm of secondary zeitgeber signals and observing the responses of the peripheral clock network. For example to approach the role of food as a secondary zeitgeber (Fig. 2) in photic entrainment, SCN clock-deficient mice could be subjected to a constant feeding paradigm, in which mice are fed every few hours such that the circadian rhythm in food intake is abolished [57]. If peripheral clocks remain aligned to the LD cycle under such conditions, it can be concluded that food signals are not necessary to synchronize peripheral clocks in SCN clock-deficient mice. Another zeitgeber candidate, glucocorticoid rhythms, could be abolished by subcutaneous implants that release corticosterone constantly over the course of the day in surgically or chemically adrenal-ablated mice [58]. To test the role of autonomic signals, optogenetic approaches may be used to manipulate circadian ANS activity and investigate the effects on peripheral clock function. Of note, with experiments such as described above, only the necessity for a single zeitgeber signal can be tested. It is likely that a real-life situation is more complicated with several, partially redundant signaling pathways contributing to synchronizing peripheral clocks. Moreover, depending on the external condition, the contribution of different pathways to peripheral synchronization may vary. Experiments in which mice are subjected to conflicting or noisy zeitgeber signals will be very informative in this context.

An alternative approach to analyze circadian network connectivity would be to abolish clock function in several tissues at the same time and investigate the response of the remaining clock network. Given the role of glucocorticoids in tissue clock regulation it might be of particular interest to disrupt, in addition to the SCN pacemaker, the adrenal clock or adrenal clock outputs in SCN clock-deficient mice, e.g. by adrenal-targeted disruption of the essential clock gene *Bmal1* [51, 59]. One hypothesis is that adrenal and SCN clocks together play a key role in synchronizing the overall circadian

network, thus expecting further deterioration of internal synchrony in adrenal and SCN clock double-deficient mice.

It should be noted that so far most genetic experiments targeting the SCN clock use a deletion of *Bmal1*. This is understandable since *Bmal1* is the only single clock gene discovered so far whose deletion results in complete behavioral arrhythmicity under constant conditions. However, combinatorial deletions of other genes – e.g. *Cry1/2* [60] or *Per1/2* [61] – also result in a loss of circadian rhythmicity. Thus, in order to clearly distinguish between (*Bmal1*) gene and circadian clock loss effects one would need to test whether manipulation of these gene pairs specifically in the SCN has similar effects. Moreover, current conditional genetics never target all cells within a tissue of interest and in, both, the Husse and the Izumo papers not all cells in the SCN are affected, thus offering the possibility that residual rhythms are retained in the mutant SCN. In line with this, Lee et al. [62], using a similar approach targeting neuromedin S-positive neurons in the SCN, have shown that even when 80% of SCN neurons lack *Bmal1* residual behavioral rhythmicity is observed for some time after mutant mice are released into DD. Finally, even in conventional – and thus complete – *Bmal1* mutant SCN slice cultures residual oscillations are observed, although with different periodicity and reduced robustness [63].

While cell culture data suggest that *Bmal1* deletion disrupts clock function at the molecular level, an alternative explanation would be that *Bmal1* knock out in the SCN may mainly affect intercellular synchrony within the SCN neuronal network. In line with this, residual oscillations of at least some clock genes are still observed in the SCN of mice with *Syt10*-CRE driven deletion of *Bmal1* [64]. Importantly, similar effects can be obtained by impairing synaptic activity in the SCN by tetanus toxin [62]. Further, a common feature of all these mouse models is an accelerated phase shifting under jetlag conditions, which is also observed when SCN intercellular coupling is affected by different means [65–67]. Taken together, further complementary studies are needed to distinguish between cellular network and molecular clock disruption in the SCN and its effects on peripheral clock gene regulation.

It will further be of importance to study the molecular endpoints of resetting signals in target tissues. For example, it has been shown that some rhythmic liver transcripts are regulated by signals derived from outside this tissue, whereas other transcripts are controlled by the local clock gene machinery [68, 69]. Studies on SCN clock-deficient mice published so far only looked at a handful of canonical clock genes in a limited number of tissues. In order to obtain a more comprehensive picture of synchronization mechanisms and physiological outputs it will be necessary to study a broader spectrum of readouts. Transcriptome studies from peripheral tissues could identify those genes that are reset independently of the SCN clock and those that require SCN pacemaker function. In combination with the above-described experiments aimed at dissecting the internal routes of entrainment such approaches will allow to specifically associate transcriptional – and, ultimately, physiological – rhythms with different synchronization signals. It is intriguing to speculate that different zeitgeber signals may act via

different mechanisms (in different tissues). For example photic signals have been shown to affect the clockwork via induction of *Per* gene expression whereas metabolic cues may signal via *Bmal1*, *Rev-erba* [70], or *Clock* [71]. Even others may exert their effect through epigenetic mechanisms [72].

## Conclusions and prospects

Recent data suggest that the organization of the mammalian multi-clock system is not strictly hierarchical. Peripheral clocks can be aligned with and, likely, entrained to external conditions in a largely “federated” fashion that conveys plasticity to the system. In the absence of periodic zeitgeber information, the clock system relies on coordinating signals from the SCN master clock, thus switching to a more hierarchical organization. This mixed modality may represent a compromise between a need for fine-tuned adaptation to complex environmental signatures and a need for circadian stability under less reliable zeitgeber conditions. The relative contribution of either mechanism might depend on the situation. With the development of more sophisticated genetic tools, we are finally in a position to start to functionally dissect circadian network organization in the living mammal and devise more specific means of manipulating clock function – for example during jetlag or in the context of various diseases.

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