The daily rhythm of mice

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ABSTRACT

The house mouse *Mus musculus* represents a valuable tool for the analysis and the understanding of the mammalian circadian oscillator. Forward and reverse genetics allowed the identification of clock components and the verification of their function within the circadian clockwork. In many cases unforeseen links were discovered between a particular circadian regulatory protein and various diseases or syndromes. Thus, this model system is not only perfectly suited to pinpoint the components of the mammalian circadian clock, but also to unravel metabolic, physiological, and pathological processes linked to the circadian timing system.

1. Introduction

The house mouse *Mus musculus* represents a well-characterized model system. It combines the ease of genetic manipulation with a relatively high reproduction rate and fast developmental progression. In addition, most of its genome is mapped and sequenced. Thus, it is not surprising that the house mouse is used also in the field of mammalian chronobiology. As such, circadian phenotypes are easy to detect. Locomotor activity or voluntary locomotor activity is measured with passive devices (implanted sensors or infrared-beam breaks) or running wheel devices, respectively [1]. Mice as nocturnal creatures are active mainly during the dark phase. This property is maintained under free-running conditions (i.e., constant darkness), when its endogenous circadian clock dictates the behavior of the animal, devoid of all external timing cues.

The circadian timing system of mammals is organized in a hierarchical manner [2,3]. A specialized center in the hypothalamus of the brain synchronizes the organism to the external photoperiod. This center, localized close to the chiasm of the two optic nerves, is called suprachiasmatic nucleus (SCN). It receives light input from the eyes and multiple auxiliary brain regions and its output establishes stable phase relationships between peripheral circadian clocks. At the base of circadian phenomena are molecular oscillators [4,5]. These cell-autonomous, self-sustained devices are based on transcriptional and post-translational feedback loops. Since circadian oscillators provide a periodicity that is not exactly 24 h (about 23.5 h in mice), the phase needs to be adjusted every day to match the external photoperiod. Due to the ability to respond to environmental signals such as light, the molecular network of the circadian oscillator is perfectly synchronized to the external photoperiod.

Specially equipped animal facilities can screen dozens, sometimes hundreds of mice in parallel for disturbances of their circadian timing system. Absence, shortened or lengthened wheel-running behavior (change in period), and alterations to adapt to changing photoperiods (phase-shifting) may indicate alterations in the circadian timing system. Starting from a forward genetics approach, the mutation responsible for a given phenotype is identified by positional cloning. Starting from a candidate gene approach, the responsible gene or its mutation is known in advance. Its effect on the circadian oscillator is analyzed. Transgenic mice are sometimes used to address specific regulatory phenomena. In contrast to e.g., tissue culture cells, the house mouse is not only suitable for research on the circadian oscillator, but also suitable to monitor the impact of mutations on the coordination of the entire metabolism and physiology.

We will start with an overview of mouse model systems and their impact on our understanding of the mammalian circadian oscillator. Then, we focus on some special mouse mutants that allow deeper insight into the functioning of the circadian clock.

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Finally, we focus on the interplay of the clock mechanism with disturbances that resemble diseases found in humans.

2. Disturbing rhythms of mice

The first mouse gene identified to be involved in the mammalian circadian oscillator was *Circadian locomotor output cycles kaput* [6] (*Clock*; Table 1). It was found in a large-scale mutagenesis and phenotype screen from *N*-ethyl-*N*-nitrosourea treated mice. The mutation was mapped by positional cloning and its phenotype could be attenuated by the incorporation of additional transgenes containing a functional *Clock* locus into the genome of mice [7,8]. The mutation arose most probably from an A to T point mutation in the 5′ splice site of intron 19. As a consequence an in-frame deletion of the entire exon 19 resulted ($^{\Delta 19)}$). This autosomal-dominant mutation provoked a lengthening of the free-running period (up to 28 h) and eventually arrhythmicity. Even if we believe nowadays that many of the phenotypes observed with these mice were due to a gain-of-function phenotype of the resulting mutant CLOCK$^{\Delta 19}$ protein, its impact on the model building of the mammalian circadian oscillator was tremendous. After the identification of CLOCK's function as a transcriptional activator operating via so-called E-box motifs [9,10], a model based on a transcriptional and post-translational feedback loop integrating positive and negative elements was proposed for mammals [11] (see Fig. 1).

Some years later, a knock out model for the *Clock* gene was established [12]. These mice had a slightly shorter period length than their wild-type controls. In further experiments it was found that NPAS2, a true homolog [13], compensates for the lack of functional CLOCK protein in the SCN [14]. Consequently, double knock out mice, by contrast to the individual single knock out mice, were behaviorally arrhythmic. Surprisingly, the CLOCK protein had a much greater impact on peripheral oscillators than NPAS2, which is most operative in the brain [15]. This indicates that within the circadian oscillator homologous proteins can fulfill tissue-specific tasks of gene expression. Most probably, due to its nature as a dominant-negative regulator with normal DNA-binding capability, the mutant CLOCK$^{\Delta 19}$ protein could interfere with the activity of both wild-type proteins and therefore provoke a striking phenotype [6–8].

To function as transcriptional activator, the CLOCK protein forms heterodimers. Its interaction partner was identified as Mop3/BMAL1 [16,17]. The proteins bind together to their cognate DNA target sequences so-called E-boxes. Single knock out mice for *Mop3/Bmal1* became immediately arrhythmic after their release into constant dark conditions [17]. Therefore, this protein appeared to have a prominent function within the circadian oscillator. The mice displayed further phenotypes like reduced activity and body weight. These phenotypes could be rescued by the tissue specific expression of *Bmal1* in the muscle, while the

Table 1
Mouse circadian clock and clock-related genes (modified from [4]).

Gene	Average CT at peak expression		Allele	Mutant phenotypes in DD	Ref.
	SCN	Periphery			
Bmal1	15–21	22–02	*Bmal1*$^{-/-}$	Arrhythmic	[17]
Clock	Constitutive	21–03	*Clock*$^{\Delta 19/\Delta 19}$	4 h longer period/arrhythmic	[6]
			Clock$^{-/-}$	0.5 h shorter period	[12]
Per1	4–8	10–16	*Per1*^{Brdm1}	1 h shorter period	[29]
			Per1^{ldc}	0.5 h shorter period	[107]
			Per1$^{-/-}$	0.5 h shorter pd/arrhythmic	[27]
Per2	6–12	14–18	*Per2*^{Brdm1}	1.5 shorter pd/arrhythmic	[26]
			Per2^{ldc}	Arrhythmic	[107]
Per3	4–9	10–14	*Per3*$^{-/-}$	0–0.5 h shorter period	[108]
Cry1	8–14	14–18	*Cry1*$^{-/-a}$	1 h shorter period	[34]
					[36]
Cry2	8–14	8–12	*Cry2*$^{-/-a}$	1 h longer period	[34]
					[35]
Rev-erbα	2–6	4–10	*Rev-Erbα*$^{-/-}$	0.5 h shorter period	[41]
				Disrupted photic entrainment	
Rorα	6–10	arr. various	*Staggerer*	0.5 h shorter period	[46]
				Disrupted photic entrainment	
Rorβ	4–8	18–22	*Rorβ*$^{-/-}$	0.5 h longer period	[47]
					[109]
Rorγ	N/A^{b}	16–20 various^{b}	*Rorγ*$^{-/-}$	Unknown	
NPAS2	N/A^{b}	0–4	*NPAS2*$^{-/-}$	0.2 h shorter period	[110]
Bmal2	Constitutive			n/d^{e}	[111]
					[112]
CK1ε	Constitutive	Constitutive	*CK1ε*$^{tau c}$	0.4 h shorter period	[113]
			CK1ε$^{-/-}$	0.3 h shorter period	[114]
CK1δ	Constitutive	Constitutive	*Csnk1δ*$^{-/+}$	0.5 h shorter period	[115]
Decl	2	14	*Dec1*$^{-/-}$	No difference in period	[116]
			Dec1$^{-/-}$	0.15 h longer period	[117]
			Sharp2$^{-/-}$	No difference in period	[118]
					[119]
Dec2	6	14	*Sharp1*$^{-/-}$	No difference in period	[118]
					[119]
Tim	12^{d}	Various	n/d^{e}	Embryonically lethal	[120]
					[121]
Fbxl3	n/d^{e}	n/d^{e}	*Fbxl3*$^{-/-}$	2–3 h longer period	[51]
					[49]
					[50]

^{a} Two independent groups generated *Cry1* and *Cry2* null mutants and the mice showed similar phenotypes.
^{b} N/A = not detected in the SCN.
^{c} Hamster mutation.
^{d} The *Tim* full-length transcript is rhythmically expressed in the SCN but not the short form [120].
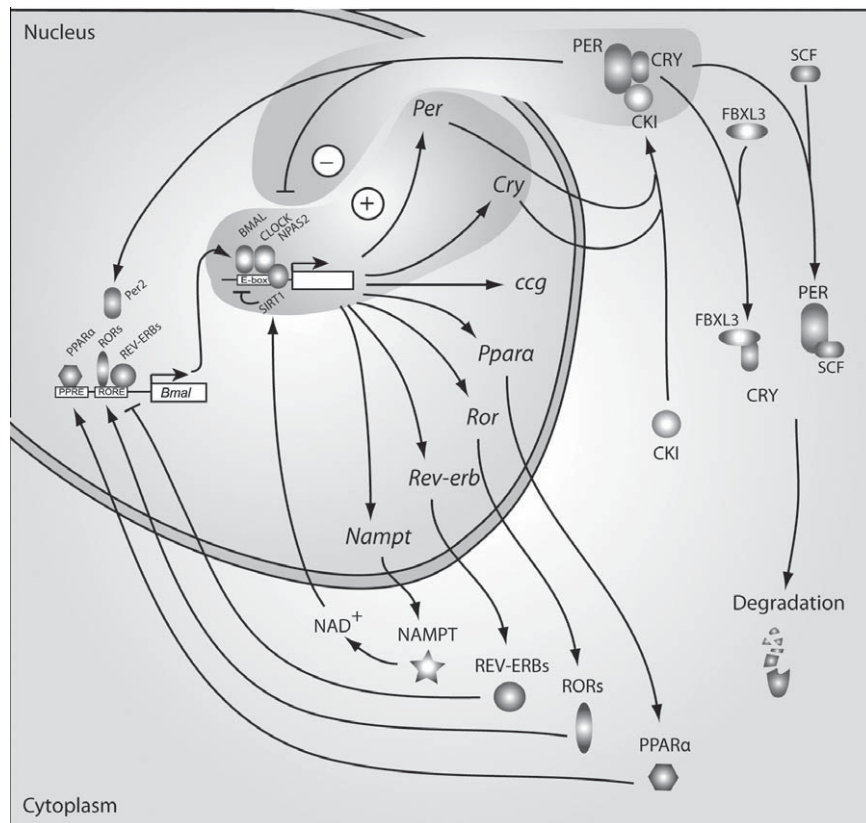^{e} n/d = not determined.

Fig. 1. Model of the mammalian circadian oscillator. Components of the core loop (BMAL and CLOCK/NPAS2) drive the expression of the *Per* and *Cry* genes. Upon reaching a certain threshold concentration, the PER and CRY proteins enter into the nucleus. This causes repression of their synthesis. As a consequence, the protein levels of the repressors decline and a new cycle of transcriptional activation and repression can occur. In concert with post-translational regulatory mechanisms depicted on the right (SCF, FBXL3, CKI and CKII), which affect the stability or the cellular localization of clock components, a period length of about a day is obtained. To the core loop a variety of other feedback loops are linked. The stabilizing loop consists of REV-ERBs or PPARα/RORs as repressors or activators of the *Bmal1* gene, respectively. The oscillator drives directly output genes (ccg), e.g., the *Nampt* gene, which regulates NAD$^+$ synthesis and may therefore rhythmically affect SIRT1 activity. A relatively new link concerns the PER2 protein, which can interact with several nuclear receptors to fine tune the expression of the *Bmal1* gene. (+) components of the positive limb of the core loop; (−) components of the negative limb of the core loop. Adapted from [4,106].

circadian phenotype was rescued solely by tissue-specific expression of *Bmal1* in the brain [18]. A similar rescue was also possible by reconstituting the expression of *Bmal1* in the SCN by injection of genetically modified adeno-associated viruses [19]. Tissue-specific knock out mice of the *Bmal1* gene in the liver had no functional oscillator in this tissue [20]. In addition, the glucose metabolism in the liver was disturbed. Consequently, these mice exhibited time-restricted hypoglycemia on the systemic level. This particular finding suggested that peripheral oscillators are directly involved in the control of rhythmic physiological processes (see below). In *Mop3/Bmal1* knock out mice, the expression of the related *Bmal2* gene was drastically down regulated [17,21]. A transgenic rescue of *Bmal2* expression in *Mop3/Bmal1* knock out mice restored rhythmic locomotor activity and the other phenotypes as well. Therefore, similar to the situation of *Clock* and *Npas2*, there may exist two functional variants of *Bmal* with redundant and non-redundant functions.

The mouse mutants discussed so far had an impact on the positive limb of the core loop of the mammalian circadian oscillator (Fig. 1). Complexes consisting of BMAL1/2 and CLOCK/NPAS2 components bind rhythmically to DNA to activate transcription. The players of the negative limb counterbalance their action. The first mouse mutants generated for these players were for the *Period 1, 2* and *3* (*Per*) genes [22–27]. The first two candidates were shown to affect the circadian oscillator. Mice deficient for these proteins displayed a shorter free-running period and in the case of PER2 became gradually arrhythmic. However, this phenotype was some-

what dependent on the design and the genetic background of the knock out mice [28]. The combination of *Per1* and *Per2* deficiency yielded mice that became immediately arrhythmic under constant dark conditions (Table 2) [29]. In addition, inducible overexpression of PER2 in the brain provoked arrhythmicity in transgenic mice [30]. Therefore, PER proteins are mandatory for the feedback mechanism of the circadian oscillator.

In analogy to the situation found within the *Drosophila* circadian oscillator, the *Per* genes were considered as negative regulators of their own expression. However, some of the phenotypes observed also suggested a positive function of PER2 on the expression of the *Bmal1* gene [11,26,29]. This observation was recently strengthened by the finding that PER2 can interact with nuclear receptors on the regulation of the *Bmal1* gene [31]. However, the main function of the PER proteins is still to repress the activity of the BMAL/CLOCK (or NPAS2) complex. Mechanistic insights were recently provided from the *Drosophila* system. Here the singular Per protein interacts with the analogous activating Cycle/Clock complex and they become detached from the DNA together [32]. This causes repression of *Per* gene transcription. A similar mechanism may be operative in mammals as well.

The PER proteins form stable complexes with the Cryptochrome (CRY) proteins, which are the other components of the negative limb [33–36]. The individual knock out of either of the two variants of these genes was quite interesting. While *Cry1*-deficient mice displayed a shorter free-running period, *Cry2* knock out mice displayed a longer period. Mice deficient for both components

Table 2
Mice with multiple mutations in clock genes.

Genes	Alleles	Mutant phenotypes in DD	Ref.
Bmal1/2	Bmal1$^{-/-}$Bmal2tg	Rhythmic	[21]
Per1/2	Per1^{Brdm1}/Per2^{Brdm1}	Arrhythmic	[29]
	Per1^{ldc}/Per2^{ldc}	Arrhythmic	[107]
Cry1/2	Cry1$^{-/-}$/Cry2$^{-/-}$	Arrhythmic	[34]
			[36]
Per1/Cry1	Per1^{Brdm1}/Cry1$^{-/-}$	Rhythmic	[37]
Per1/Cry2	Per1^{Brdm1}/Cry2$^{-/-}$	Rhythmic <6 months, arrhythmic >6 months	[37]
Per2/Cry1	Per2^{Brdm1}/Cry1$^{-/-}$	Arrhythmic	[39]
Per2/Cry2	Per2^{Brdm1}/Cry2$^{-/-}$	Rhythmic	[39]
Per1/Cry1/2	Per1^{Brdm1}/Cry1$^{-/-}$/Cry2$^{-/-}$	Arrhythmic, reduced activity, no breeding	[38]
Per2/Cry1/2	Per2^{Brdm1}/Cry1$^{-/-}$/Cry2$^{-/-}$	Arrhythmic, reduced activity	[38]
Per1/2/Cry1	Per1^{Brdm1}/Per2^{Brdm1}/Cry1$^{-/-}$	Arrhythmic, reduced activity, no breeding	[38]
Per1/2/Cry2	Per1^{Brdm1}/Per2^{Brdm1}/Cry2$^{-/-}$	Arrhythmic, reduced activity, only 2 litters	[38]
Per1/Rev-erbα	Per1^{Brdm1}/Rev-Erbα$^{-/-}$	Rhythmic, high amplitude resetting	[122]
Per2/Rev-erbα	Per2^{Brdm1}/Rev-Erbα$^{-/-}$	Rhythmic, can switch to arrhythmic and back	[31]

became immediately arrhythmic similar to the *Per1/Per2* double knock out mice (Table 2). Interestingly, certain combinations of single knock out mice of *Per* and *Cry* genes can have more or less severe phenotypes [37–39]. This can be explained by complex formation of PER and CRY proteins with different affinities and activities. However, the free-running behavior of a knock out mouse may be misleading. Intercellular coupling of SCN neurons can mask the effect of a gene knock out [40]. For example, deficiency for *Per1*, *Cry1*, or *Cry2* has only a mild impact on an intact SCN but a severe impact on dissociated SCN neurons. Hence, intercellular coupling can provide robustness to the circadian oscillator against perturbations that are due to the lack of an oscillator component.

Coupled to the core loop is a second, stabilizing loop (Fig. 1). Mice deficient for the nuclear receptor REV-ERBα showed a shorter period probably due to dampened amplitude of *Bmal1* expression [41]. The REV-ERBα protein bound to the promoter region of the *Bmal1* gene in the phase of transcriptional repression [31]. This specific action of REV-ERBα was exploited to shut down specifically the circadian oscillator in the liver [42]. Inducible overexpression of this nuclear receptor completely abolished *Bmal1* expression and consequently the rhythmicity of the circadian oscillator. Surprisingly, some genes were rhythmically expressed in such livers, including the *Per2* gene. These rhythmic gene expressions were demonstrated to rely on systemic cues. A function for the related *Rev-Erbβ* gene on the circadian oscillator was not yet examined in vivo. In tissue culture cells it behaves very much like the *Rev-Erbα* gene [43].

In contrast to the negative regulator REV-ERBα, the nuclear receptors RORα and PPARα were important for the transcriptional activation of the *Bmal1* gene in the SCN and the liver, respectively [44–46]. Other related nuclear receptors like RORβ and PPARγ had also an impact on the circadian oscillator or *Bmal1* expression [43,47,48]. Nuclear receptors hence target preferentially the stabilizing loop. The precise functions of the stabilizing loop, however, remain to be determined. In mice, rhythmic expression of the *Bmal1* gene e.g., in the liver appears unnecessary for core oscillator function and circadian rhythm generation [31]. However, in the SCN with constant high expression of BMAL1, there was a yet to be determined impact on the free-running behavior observed.

Taken together, the various genetic mouse models were very valuable to unravel the operating mode of the mammalian circadian oscillator (Fig. 1). The transcriptional activators BMAL/CLOCK (or NPAS2) activate transcription of the *Per* and *Cry* genes via E-box motifs. Upon reaching a certain threshold concentration, the negative components feed back onto their own synthesis. When the protein concentrations finally decline, repression is released and another cycle of about a day can occur. Problems arising with the interpretation of circadian phenotypes are due to the fact that

all the relevant components of the oscillator structure exist at least in duplicates. There may consequently be redundant and non-redundant functions for each set of components. As a conclusion, further investigations are necessary to assign precise functions to each component and to understand the clockwork of the circadian oscillator. An interesting avenue in this context is the study of epigenetic regulation of gene expression reviewed in this issue by Merrow and Ripperger.

3. Lessons from mice

Forward genetics, as mentioned above, provides information on the phenotype, the responsible mutation and sometimes allows drawing conclusions about the function of the protein involved. Recently, two large-scale mutagenesis projects identified two independent mutations in the same protein [49–51]. The FBXL3 protein is an E3-type ubiquitin ligase. The two independent mutations, provoking very long free-running periods of about 27–28 h of homozygous animals, mapped to an interaction domain for CRY proteins. Indeed, due to the lack of interaction, the CRY proteins were less ubiquitinylated and hence became stabilized. Surprisingly, on the molecular level, the expression of the *Per* genes was down-regulated, provoking long periods due to prolonged repression by CRY proteins [51].

Interesting insights in further post-translational regulatory phenomena were obtained by generating mice with a human transgene for the *Per2* S662G mutation [28]. These mice could phenocopy the *familial advanced sleep phase syndrome* observed in certain families. The mutation of serine 662 to glycine inactivates the first phosphorylateable serine in a phosphorelay located in this region of the PER2 protein. The phosphorylation of serine 662 greatly facilitates phosphorylation of serine 665, 668, 671, and 674 by Casein kinase I (CKI). Mice with the human transgene and this particular mutation displayed a drastically shorter free-running period length. Surprisingly, when a transgene was introduced into mice with a serine 662 to aspartic acid substitution, a longer period length resulted, indicating that the phospho-mimicking amino acid allowed for normal CKI function on the phosphorelay. Transferring the different transgenes into heterozygous *CkIδ* knock out mice supported the hypothesis that CKI was involved in the subsequent phosphorylation of the phospho-relay (see contribution in this issue by the Kramer lab).

The strength of transgenes, however, is the direct visualization of gene expression in living mice. Two kinds of reporters are commonly used, luciferase reporters and fluorescent protein reporters. Amongst the luciferase reporters two different and complementary systems are quite popular. The first system drives luciferase expression from circadian regulatory regions. One of the first

transgenic reporter mouse used in chronobiology had integrated the *mPer1*-promotor driving circadian luciferase expression [52]. A 7.2 kilobase pair (kbp) fragment of the mouse *Per1* gene containing its two alternative promoters in front of exon 1A and 1B was fused to the firefly luciferase reporter gene (a similar mouse line was generated with 6.75 kbp of *mPer1* regulatory region [53]). The distribution of expression of this transgene resembled the endogenous *mPer1* expression and a light pulse could induce expression of the luciferase reporter in the SCN. Brain slices of the SCN region displayed rhythmic luciferase activity in culture. Therefore, expression of the transgene matched perfectly with the endogenous gene.

The system was later on used to monitor circadian gene expression in a living mouse [54]. An optic fiber cord linked to a photomultiplier device was placed just above the SCN. Upon constant perfusion of luciferin, the lumigenic substrate for the luciferase enzyme, into this brain region, circadian luciferase activity was monitored for up to five days in free-running mice. Altogether, these mice represent an elegant, well-controlled experimental system to monitor circadian gene expression in living animals.

These reporter-dependent systems allow the visualization of circadian rhythms in isolated peripheral organs. A transgenic rat with the incorporation of a similar *Per1*-driven reporter was used to address the impact of feeding on peripheral oscillators [55]. Mice as nocturnal animals prefer to consume food in the dark phase. When they become challenged by a temporarily restricted food access, their peripheral circadian oscillators adapt to this new situation. For the experimental procedure, the access of food was restricted to some hours during the light phase. The mice displayed food anticipatory activity, i.e., they became active a couple of hours before the food was administered. Surprisingly, there occurred an uncoupling of the SCN oscillator from the peripheral oscillators under these conditions. While the SCN clock was still synchronized to the environmental light-dark rhythm, the phase of the liver and lung clocks changed by up to 12 h. As a conclusion, timing cues like food uptake are thought to synchronize peripheral oscillators. However, the central SCN circadian clock governs feeding behavior itself.

The second system relies on a fusion protein between PER2 and luciferase [56]. This was achieved by an in frame knock in of the luciferase open reading frame into the last exon of the mouse *Per2* gene. This fusion protein was sufficient to drive circadian rhythms in mice. The advantage of this system is that the luciferase activity reflects directly the presence of the PER2 protein. When compared to a mouse strain in which a mouse *Per2* gene promoter drives luciferase expression, the typical delay of PER2 protein accumulation measured up to mRNA accumulation was conserved [57].

The *Per2:luc* mice were successfully utilized to investigate the function of the SCN within the circadian clock system. Tissue explants from multiple origins (liver, lung, etc.) showed free-running circadian rhythmicity albeit with different period lengths. These rhythms were nearly as robust as the rhythms of luciferase activity originating from SCN tissue. Shortly after the removal from the body context, the phases of PER2:Luc expression in essentially all the tissues examined were coupled. In contrast, in genetically identical animals with an ablation of the SCN, all the tissues displayed random phases of PER2:Luc expression. Therefore, the main function of the SCN is to organize stable phase relationships between the peripheral oscillators (see contribution by the Kalsbeek lab in this issue).

In a similar approach, intercellular coupling of SCN neurons was demonstrated using the *Per2:luc* mice [40]. This reporter was crossed into various knock out mice. Most mice (apart from the arrhythmic *Cry1/Cry2* double knock out mice) displayed circadian rhythms in explanted SCN tissues. However, there were differences observed. For example, SCN tissue from *Cry1*- or *Per1*-deficient mice displayed synchronized luciferase activity. When the SCN neurons were dispersed, essentially all of the neurons lost rhythmicity over a couple of days. Therefore, the culture became rapidly desynchronized. The phenomenon of intercellular coupling can obstruct the impact of a clock mutation [40].

In essence, the same kind of experiments with luciferase-based reporter lines can be performed also with fluorescence protein-based reporter lines. Due to the characteristics of fluorescent proteins, it is possible to monitor circadian rhythms in individual living cells. The Green Fluorescence Protein (GFP) has one major draw back. By contrast to luciferase, the GFP protein, after appropriate folding into a barrel-like structure, is extremely stable (with a half-life of more than a day). Therefore, this protein is not suited to monitor the highly dynamic processes that occur within the mammalian circadian oscillator. This draw back was overcome by fusing various degradation motifs to the protein. Nowadays variants of GFP exist with different spectral excitation and emission spectra with half-lives in the range of 1–2 h.

This system was used to demonstrate the behavior of the circadian oscillator during cell division in vitro [58]. Destabilized Venus (a yellow-shifted variant of GFP) was fused into exon 3 of the mouse *Rev-Erbα* gene in a genomic construct. Mouse NIH 3T3 fibroblasts were stably transfected with this construct and after synchronization with dexamethasone the accumulation of the Venus-reporter monitored in real-time. Surprisingly, cell-autonomous oscillations were detected in all cells of the culture albeit with individual phases and period lengths. These rhythms could be synchronized by a dexamethasone shock. In addition, using this system it became obvious that oscillator information was passed to the daughter cells after cell division. An independent line of experiments used single-cell luciferase monitoring of stably transfected RatI cells. The obtained results were essentially the same [59].

Recently, fluorescent reporters were incorporated into the genome of mice [60]. Two different kinds of fluorescent reporters were used to follow the expression of *Per1* and *Per2* in the brain, a Venus reporter and a RED fluorescent protein, respectively. The expression of the transgenes was driven from the entire authentic promoters and regulatory regions. Again, transgene expression matched the expression of the endogenous gene. Surprisingly, in the SCN a different localization of the two different reporters was observed. In other brain regions, these differences were even more drastic. Venus was visible in neuronal and non-neuronal cells, while RED was more restricted to glial cells and some progenitor cells of the dentate gyrus. Therefore, there are tissue-specific differences in the accumulation of the PER1 and PER2 proteins as well. Taken together, we learned a lot about the properties of the mammalian circadian oscillator from transgenic mice.

4. The impact of clock genes on physiology

The above paragraphs describe the effects of mutations in clock genes mainly on activity as a behavioral read-out for the circadian clock. Interestingly, mutations in and deletions of clock genes affect a plethora of other physiological processes as well. This indicates that either the circadian clock is important for these processes or that clock genes might have also clock-independent functions.

One of the first observations that a clock gene has an impact on physiology apart from clock function was made in mice mutant in the *Per2* gene. These mice are more prone to develop a form of cancer in response to gamma radiation, which is accompanied by altered expression of genes involved in cell cycle regulation such as *Myc*, cyclins and *Mdm2* [61]. It later appeared that the circadian clock is important in the timing of the cell division cycle in mice by directly regulating *wee1* gene expression [62]. However, not all

clock components have the same effect on cancer development. A mutation in the *Cry* genes in mice sensitized for cancer development due to a p53 mutation protected animals from early onset of cancer [63]. Mice with a functional deficiency of *Clock* do not display predisposition to tumor formation both during their normal life span or when challenged by gamma-radiation [64]. The above results illustrate the dichotomy in biological consequences of the disruption of the circadian clock with respect to cancer development. There are probably complex interconnections between carcinogenesis, ageing and the individual components of the circadian clock because nucleotide excision repair has been suggested to be under clock influence [65,66] and DNA damage affects phase resetting in the murine circadian clock [67].

Feeding has been observed as the dominant Zeitgeber for many organs [55,68] indicating that nutrition affects the clock. *Clock* mutant mice display obesity and metabolic syndrome highlighting a function of the circadian clock in co-ordination of food uptake and processing [69]. Since the circadian clock regulates the rate-limiting enzyme of mammalian nicotinamide adenine dinucleotide (NAD$^+$) biosynthesis, nicotinamide phosphoribosyltransferase (NAMPT, see Fig. 1), and as a consequence causes levels of NAD$^+$ to display circadian oscillations [70,71], the clock affects at least those metabolic pathways that depend on NAD$^+$ or NADH, respectively. Another proposed interaction between metabolic cycles and the circadian clock appears to be an enzyme that responds to nutrient availability, adenosine monophosphate-activated protein kinase (AMPK). AMPK phosphorylates the clock protein CRY1 thereby marking it for degradation via Fbxl3 [72], directly affecting the circadian oscillator. An elegant study by Kornmann et al. [42] showed that PER2 is not only part of the circadian oscillator affecting metabolism, but that it also responds to systemic cues, thereby linking the clock and metabolism in an interdependent fashion. This might be achieved partially through protein-protein interactions, such as PER2-nuclear receptor binding [31]. There is also evidence for REV-ERBα affecting aspects of metabolism. Sterol response element binding protein (SREBP) regulated pathways are affected in *Rev-erbα* knock out mice leading to changes in temporal lipid accumulation in blood and liver as well as alterations in bile acid accumulation [73]. Lipid metabolism appears also to be regulated by PER2 through direct interaction with PPARγ [123]. Additional support for a strong relationship of the clock with metabolism comes from a recent genome-wide profiling study showing that most of the BMAL1 targets appear to be related to metabolism [124].

Why should metabolic cycles be linked to circadian cycles? One of the important functions of the circadian clock is to allow an organism to predict recurring daily events. In a competitive environment such as our world this gives a competitive advantage since the body is prepared for e.g., food uptake allowing efficient and rapid uptake of nutrients. In support of this view are competition experiments performed with photosynthetic cyanobacteria [74]. Strains having a clock that is in resonance with the environmental light-dark cycle outcompete strains not in resonance. Hence, the circadian clock might also be important to predict food availability in mammals. In line with this hypothesis is the finding that mice with a mutation in the *Per2* gene do not have food anticipatory activity (FAA) and temperature increase [75] indicating that PER2 is involved in food anticipation either in its function as a clock gene or via a clock-independent mechanism. Although other clock components such as NPAS2 or Mop3/BMAL1 show reduced food anticipation [19] recent experiments suggest that daily rhythms of FAA do not require the circadian clock [76].

Glucose is an important energy source in mammals but due to its ability to chemically react with lipids and proteins it has to be kept constant at low levels in the blood (at around 6 mM in humans). Several clock components affect glucose levels in the mouse

such as *Clock* and *Bmal1* [20,69] indicating an involvement of the clock in the regulation of blood glucose levels (see also contribution by the Kalsbeek lab in this issue). However, the circadian clock would impose a 24-h cycling rhythm on these glucose levels. To achieve homeostatic levels the central clock regulates via rest-activity cycles food consumption and the liver clock in an opposite cycling manner expression of GLUT2, the glucose uptake transporter in liver cells [20]. As a consequence glucose uptake in the liver becomes higher after food has been digested. When no food is taken up less glucose transporter is present on liver cells with the consequence of reduced glucose uptake. This mechanism adjusts the levels of blood glucose and hence the level over 24 h is more or less constant with a minimal amplitude of cycling [77]. This leads to a constant supply of glucose to the brain.

Light is the most powerful synchronizer of the mammalian circadian system and affects initially the brain. It is perceived mainly by retinal ganglion cells (RGCs) [78] and causes activation of signaling pathways in the SCN that converge on the phosphorylation of cAMP response element-binding protein (CREB). Phospho-CREB homodimers bind to the promoters of the clock genes *Per1* and *Per2* thereby activating their expression. As a consequence behavioral activity rhythms in mice and humans are phase advanced or delayed, depending on the time of nocturnal light exposure (reviewed in [79]).

Recent findings indicate, that light mediated signaling involving the clock gene *Per2* affects synaptic efficiency in mice by regulating the presence of vesicular glutamate transporter 1 on a defined vesicular pool [80,81]. The light regulated membrane traffic of neurotransmitter transporters may allow the presynaptic terminals to replenish during physiological rest periods and to avoid prolonged or repeated periods of enhanced stimulation. This is probably one of several reasons why mice bearing a mutation in the *Per2* gene show alterations in mood related behaviors such as addiction to cocaine [82] and consumption of ethanol [83]. Proteomic analysis revealed that synaptic vesicle cycling itself is probably important for sustaining the circadian clock in the SCN [84]. This is supported by the finding that a mutation in the GTPase Rab3, a regulator of synaptic vesicle transport and Ca^{2+}-triggered vesicle release probability, accelerates the clock by about 2 h [85]. Taken together synaptic vesicles appear to play an important role in light mediated effects on the circadian clock and its neurophysiological outputs.

Mice with a point mutation in the gene *Clock* display increased excitability of dopamine neurons, cocaine reward, and expression of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis [86]. Furthermore, these animals display a mania like phenotype similar to that observed in patients with bipolar disorder [87]. A molecular link between the circadian clock mechanism and dopamine metabolism has been established [88]. In mice, the clock proteins BMAL1, NPAS2, and PER2 directly regulate expression levels and activity of monoamine oxidase A, an enzyme important in dopamine degradation, in brain regions relevant for reward and mood related behavior. Taken together these findings indicate an involvement of the clock in the regulation of metabolism of neurotransmitters in the brain. However, it is unclear how light can affect neurotransmitter metabolism via clock mechanisms.

Blood pressure and heart rate are known to show a circadian pattern. Mice with altered core clock genes *Bmal1* and *Clock* display a disruption in this circadian pattern [89]. These mice also appear to be more resistant to immobilization stress indicating that the vascular clock may modulate the capacity to respond to environmental stressors. Interestingly, *Bmal1* and *Clock* mutant mice show a loss of vascular adaptation and predisposition to thrombosis [90], both hallmarks of endothelial dysfunction. In line with these observations is the finding that mice with a mutation in the *Per2* gene display altered vascular endothelial function due to a decreased

production of nitric oxide and vasodilatory prostaglandins as well as an increased release of COX-1-derived vasoconstrictors in aortic rings [91]. Further evidence of involvement of the *Per2* gene in the vasculature is the observation that a mutation in this gene causes Akt-dependent senescence and impairs ischemia-induced revascularization through the alteration of endothelial progenitor cell function [92].

A temporal map of the nuclear receptor transcriptome provides clues concerning the circadian control of cardiovascular physiology [93]. Nuclear hormone receptors such as RARα and RXRα can regulate CLOCK and NPAS2 activity resulting in a repression of CLOCK/NPAS2:BMAL1 mediated transcription in vascular cells [94]. Binding of retinoic acid to these receptors can phase shift *Per2* mRNA rhythms in vivo and smooth muscle cells in vitro indicating an interplay in cardiovascular function of nuclear receptors with the circadian clock. Modulation of this process via PER2 can be envisaged [31] and additional nuclear receptor agonists may be involved in the circadian regulation of vascular tone.

There is evidence that the circadian clock influences the immune system. Mice with a loss of function mutation in the *Per2* gene display a loss of interferon-γ (IFN-γ) mRNA cycling in the spleen [95]. Additionally, these animals are also more resistant to lipopolysaccharide-induced endotoxic shock than wild type mice and show decreased levels of pro-inflammatory cytokines in the serum. The impaired IFN-γ production is attributable to a defect in natural killer cell function [96]. Furthermore, *Bmal1* appears to regulate the development of B-cells [97] and in macrophages a circadian clock controls inflammatory immune responses [98]. Interestingly, IFN-γ affects electrical activity and clock gene expression in SCN neurons [99] and tumor necrosis factor alpha (TNF-alpha) suppresses expression of clock genes by interfering with E-box mediated transcription [100] depending on calcium and p38 MAP kinase signaling [101]. These findings indicate a feedback regulation between the circadian and the immune system.

Hematopoietic stem cells (HSCs) circulate in the blood stream and exhibit robust circadian fluctuations, peaking 5 h after light onset with a nadir in the dark. These circulating HSCs and their progenitors fluctuate in anti-phase to the expression of the chemokine CXCL12 in the bone marrow microenvironment [102]. Both circulating HSC levels and CXCL12 expression are regulated by adrenergic stimulation regulated by the SCN. Interestingly, bone formation occurs in mice in a diurnal manner, with the greatest remodeling occurring during the periods of light, which corresponds to the resting period [103]. Many hormones affecting skeletal mass such as parathyroid hormone and leptin undergo circadian cycling [104]. Also clock genes such as *Per2* in osteoblasts inhibit bone formation, and in their absence, leptin-driven adrenergic stimulation has a proliferative effect on osteoblasts [105]. Interestingly, Cry2 influences bone formation affecting osteoclasts, indicating that Per2 and Cry2 balance bone formation via different pathways [125]. Taken together, it appears that the circadian clock affects bone and bone marrow formation and hence will influence blood-cell production.

5. Perspectives

Mice have been very valuable for our understanding of the mammalian circadian oscillator over the past decades. Significant progress was made with the advance of new knock out mouse and mutant mouse strategies. Tissue-specific knock out mice will be necessary to unravel the link between the circadian oscillator and metabolic or physiological processes in a given tissue or the entire animal. Although there is now tremendous competition from in vitro-tissue culture systems, the overall picture can only be deduced from the specifics of mice. In the future, it will be possible to translate the results found with the house mouse to the clinic. For many typical human diseases there exist reasonably well-established mouse models. Due to the ease of these models, it will be possible to find links between disturbances of the circadian oscillator and a particular disease. Therefore, research on the house mouse will prosper for many years to come.

Acknowledgements

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