The mammalian circadian clock
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Organisms populating the earth are under the steady influence of daily and seasonal changes resulting from the planet's rotation and orbit around the sun. This periodic pattern most prominently manifested by the light–dark cycle has led to the establishment of endogenous circadian timing systems that synchronize biological functions to the environment. The mammalian circadian system is composed of many individual, tissue-specific clocks. To generate coherent physiological and behavioral responses, the phases of this multitude of clocks are orchestrated by the master circadian pacemaker residing in the suprachiasmatic nuclei of the brain. Genetic, biochemical and genomic approaches have led to major advances in understanding the molecular and cellular basis of mammalian circadian clock components and mechanisms.

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Introduction
Persistent exposure to jetlag, shift work and short winter days in northern latitudes can cause disruption of the circadian clock [1,2]. What is the circadian clock and what is its purpose? The circadian clock prepares the body for tasks that typically occur in the course of a day. For predator animals to successfully chase after prey requires that energy-generating organs and muscles are primed for peak performance at the time of the hunt. It thus makes sense that a broad spectrum of physiological parameters including the sleep–wake cycle, hormone secretion (e.g. adrenocorticotropic hormone and cortisol), heart beat, renal blood flow and body temperature fluctuate with a period of ∼24 hours. To stalk prey, the hunter’s alertness (via its sense organs and brain) and agility (via its muscles and skeleton) need to be simultaneously optimized. This coordination of preparedness for function is immensely aided by the remarkable finding that there are circadian clocks in many organs [3] and cells that are ultimately coordinated by the clockwork of the suprachiasmatic nucleus (SCN) that is located in the ventral hypothalamus (Figure 1c) [4]. The SCN integrates signals from the visual system (e.g. the light–dark cycle) and from the periphery. Although most humans in our industrialized society have been affected by the side effects of circadian clock disruption, our understanding of circadian clock mechanisms is chiefly based on animal studies; hence this brief review of recent advances in this fascinating and very active field concentrates on work with animals, mostly mice. We do, however, comment on a small number of studies in humans that have reached the level of a ‘molecular explanation’. The commonly used experimental strategies for studying circadian clocks in mammals are explained in Figure 1.

Components of the clockwork
Over the past few years, the molecular models of the circadian clock have evolved as additional clock genes have been identified (for recent reviews, see [5–10]). The central circadian clockwork consists of interwoven positive and negative feedback loops, or ‘limbs’ (Figure 2). The positive limb involves CLOCK/BMAL1 heterodimers, two basic helix–loop–helix transcriptional activators that bind to E-boxes located in the regulatory region of the period (per) and cryptochrome (cry) genes. CRY and PER proteins form oligomers that are transported from the cytoplasm to the nucleus. When located in the nucleus, CRY and PER repress their own transcription by inhibiting CLOCK/BMAL1 (negative limb). Furthermore, the positive and negative limbs are interlaced: CLOCK/BMAL1 heterodimers also induce the expression of the nuclear orphan receptor REV-ERB, which in turn represses the transcription of Bmal1 through direct binding to a REV-ERB response element in the Bmal1 promoter.

In addition to transcriptional regulation, the circadian clock is also regulated by posttranslational mechanisms. One model, mainly based on studies in cell culture [11], posits that PER protein shuttles between nucleus and cytoplasm. Cytoplasmic casein kinase Ie (and probably also other kinases) phosphorylates PER (see below) and phospho-PER becomes degraded by the proteasome.
machinery. Counteracting such degradation is CRY protein in the nucleus that binds to PER and prevents it from leaving the nucleus. Thus there is a check and balance of positive and negative regulation and the 24-hour periodicity of the circadian clock results from a combination of positive and negative transcriptional feedback, nuclear–cytoplasmic shuttling of PER, and phosphorylation and degradation of PER.

In humans, Per genes are implicated in circadian disorders [12,13,14]. In the case of familial advanced sleep phase syndrome (FASPS), the serine at position 662 of PER2 is mutated to a glycine [13]. Individuals suffering from this disorder go to sleep ~4 hours earlier than average but awake well before dawn [12]. Overall, the period length (τ) of the internal clock of these patients is ~1 hour shorter compared to that of normal people. It was found that the serine to glycine mutation maps to a region where PER2 protein binds to casein kinase Iε which can phosphorylate PER2 in vitro. In its phosphorylated form serine 662 is part of a recognition site for this kinase and is required for successive phosphorylation of other proximal serine residues. Mutation of this recognition site thus results in hypophosphorylation of human PER2 protein which, in turn, would lead to less efficient degradation of PER protein in the cytoplasm leading to an increase of its

![Figure 1](https://example.com/fig1.png)

**Figure 1**

Experimental strategies to study the circadian clock in mice. (a) A mouse is placed in a cage containing a running wheel. Running activity is measured by counting the revolutions of the wheel. Locomotion in constant darkness (‘free running’) is a read-out of the circadian clock. (b) Plotting wheel running activity for consecutive days produces an actogram revealing that locomotor activity is periodic and even in darkness regular for many weeks. Actograms are double plotted and aligned so that two consecutive days locate to a horizontal line (e.g. day 1 left and day 2 right). The red line drawn passes through the points of daily onsets of wheel running activity. The spacing between the red lines of the double plot corresponds to the circadian period length (τ). (c) Coronal section through a mouse brain. Genes of the circadian clock are expressed in the suprachiasmatic nuclei (SCN). (d) Expression levels of circadian clock genes change with a periodicity of τ even in free-running conditions (constant darkness). Such changes in gene expression are used as molecular markers to monitor circadian clock activity. (e) A light pulse administered during the activity phase (night) alters onset of the subsequent locomotor activity cycles. A light pulse applied in the early part of the night (blue arrow in left panel) delays onset of the subsequent activity cycle resulting in a ‘phase delay’ characterized by a negative Δφ (phase angle difference). By contrast, a light pulse applied in the late portion of activity phase induces phase advances (positive Δφ; right panel). This change in phase serves as read-out for how light entrains (resets) the clock. A useful measure of entraining is also a forward or backward shift of temporal expression profiles of clock genes.
nuclear concentration affecting the timing of the negative feedback loop.

The \textit{tau} mutation in hamster is caused by a single amino acid change (arginine to cysteine) at position 178 of casein kinase I\(\epsilon\) [15]. This shortens the period to \(~2\) hours in homozygous animals. Although mutant casein kinase I\(\epsilon\) still binds PER1 and PER2, phosphorylation of these clock proteins is diminished. \textit{Per} expression in the SCN of \textit{tau} animals is reduced. In addition, the decline of \textit{Per} transcript levels in the SCN occurs \(~4\) hours earlier in \textit{tau} animals consistent with the shortened period length. This more rapid decline in \textit{Per} expression indicates that the autoregulatory negative feedback of PER on its own synthesis (Figure 2) commences earlier. As in the case of FASPS, this can be understood in terms of hypophosphorylation and hence reduction of cytoplasmic degradation of PER.

\textbf{Clock input and output}

The elaborate design of the circadian clockwork residing in the SCN permits acceptance of input from the environment and production of output signals to peripheral clocks. The mechanism of light input is the subject of intense research but obviously involves the retina because eye loss in both humans and mice abolishes light entrainment. It had been shown that mice lacking cones or both rods and cones can still be entrained [16], suggesting that there are additional photoreceptors that mediate the effect of light on the circadian clock. In fact, it is now clear that light signal can be received by retinal ganglion cells whose axons form part of the retinohypothalamic tract that projects directly into the retinorecipient region of the SCN [17**,18**]. Synaptic transmission between ganglion cells and SCN neurons is mediated by glutamate and PACAP (pituitary adenylate cyclase activating peptide). SCN neurons respond to the neuronal signal by induction of \textit{per} genes through a cAMP-responsive element located in the \textit{per} promoter [19–21]. What is the light-sensing mechanism in the retinal ganglion cells? It has recently been found that a subset of retinal ganglion cells expresses the photopigment melanopsin [18**,22]. These light-responsive retinal ganglion neurons project to the SCN and to other brain regions implicated in photic entrainment [17**,18**]. Moreover, the physiologic

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**Figure 2**

Diagram of the circadian clock mechanism. CLOCK (yellow) and BMAL1 (pink) proteins (positive limb) drive the expression of \textit{Per}, \textit{Cry} and \textit{Rev-erb} genes in the nucleus. PER (red) and CRY (light green) proteins in the nucleus inhibit CLOCK/BMAL1 action by a yet unknown mechanism and thereby down-regulate their own expression and that of \textit{Rev-erb} (dark green). When REV-ERB protein is absent, \textit{Bmal1} (and possibly also \textit{Clock}) genes are derepressed and hence transcribed to produce new CLOCK/BMAL1 transcription factors that reinitiate a new circadian cycle. Clock proteins are posttranslationally modified; casein kinase I\(\epsilon\) (CKI\(\epsilon\)), for example, phosphorylates PER2. Hyperphosphorylation of PER2 decreases its stability and thus promotes its degradation. A typical circadian cycle would begin with activation of \textit{Per} (and \textit{Cry}) transcription by CLOCK/BMAL1 in the early morning. Transcript levels peak around noon and protein levels in the cytoplasm reach the zenith \(~2\) hours later. PER shuttles between the cytoplasm and the nucleus. In the cytoplasm, it is degraded following hyperphosphorylation and in the nucleus it is complexed with CRY and thereby blocks CLOCK/BMAL1 function resulting in termination of \textit{Per} and \textit{Cry} transcription. At some point when much PER is degraded in the cytoplasm, PER concentration in the nucleus is too low to keep up negative feedback and the cycle reinitiates.
properties of the melanopsin-positive ganglion cells (spectral tuning and slow response to light) resembles those reported for light-induced entrainment. Mice lacking the melanopsin gene (Opn4) have been made and analyzed for photic entrainment with the result that there is no major effect on circadian activity [23*,24*] or the induction of the light-responsive gene c-fos in the SCN [24*]. There is, however, a statistically significant reduction of the magnitude of the clock delay induced by a light pulse applied in the early night. Because Opn4 mutant mice show a less dramatic effect than one would expect if melanopsin were the sole photopigment, these studies suggest the existence of additional photopigments contributing to light entrainment.

Studying the mechanisms by which the circadian clockwork confers timing information to the body is an emerging field. Locomotor rhythms are controlled, at least in part, by diffusible secreted molecules [25] and recent work raises the possibility that TGFζ [26**] and prokineticin 2 (PK2) [27**] are such factors. TGFζ is expressed in a circadian fashion in the SCN of hamster. Moreover, application of TGFζ into the third ventricle completely blocked wheel-running activity. A major target region of the SCN and hence of secreted TGFζ is the subparaventricular zone that expresses epidermal growth factor receptor through which TGFζ exerts its effects. A hypomorphic allele of EGFR termed waved-2 causes increased wheel-running activity during the day and an imprecise onset of nocturnal activity. Another candidate clock output signal is PK2 that is expressed in the SCN in a circadian fashion [27**] and is directly regulated by CLOCK/BMAL1 heterodimers via an E-box in the PK2 promoter. This is reminiscent of arginine-vasopressin that is also a candidate clock output signal [28]. Furthermore, the circadian pattern of PK2 expression is shifted following light-induced resetting of the circadian clock. PKR2, the receptor for PK2, is present in the SCN and in many target regions of SCN efferents. Normally, PK2 expression peaks in the morning and is very low during the night. When injected into the lateral ventricle during the night, wheel-running activity is suppressed resembling that observed during the day. Thus PK2 meets criteria postulated for a circadian output gene [27**]. Nonetheless, it will be important to determine whether PK2 and PKR2 loss-of-function mutant mice show strong behavioral phenotypes as would be predicted from the current work. It is interesting to note that PKR2, a G-protein coupled transmembrane receptor, is also expressed in the SCN [27**], raising the possibility that this ligand/receptor system is involved in interneuronal communication and possibly in synchronization of SCN neurons. The relationship between PK2 and TGFζ merits further study. Because EGFR but not PKR2 is expressed in the subparaventricular zone, the two secreted factors may control different components of the circadian clock output.

Circadian clocks are found in many places

When the expression pattern of Per1 was first described, it was found that its transcript levels were oscillating not only in the SCN but also in the pars tuberalis and the retina [29]. Balsalobre et al. [30] made the striking observation that serum shock induced circadian gene expression in cultured mammalian cells. The initial observation of the existence of clocks in many cell types and organs has now rapidly expanded and led to the view that mammals have numerous peripheral clocks. A useful approach to study clocks in peripheral organs is provided by transgenic mice [31] or rats [32] in which the Per1 promoter drives a luciferase reporter gene. This allows one to directly and continuously measure Per1 promoter activity that, at first appearance, parallels endogenous Per1 expression (but see [31]). Using this technology, it was shown that liver, lung, skeletal muscle and many brain regions exhibit circadian reporter gene expression. These findings are bolstered by direct measurements of mRNA encoding the clock constituents (e.g. [33**]).

What controls the rhythm of peripheral clocks? The SCN has long been viewed as a master regulator imposing its rhythm onto the ‘slave’ clocks of the periphery (e.g. see [34]). In other words, the SCN was thought to be at the top of a hierarchically organized circadian system. The current view of the SCN is somewhat different in that the SCN would not directly force rhythm on all peripheral clocks (e.g. those in liver) but rather act as a reference clock used by peripheral clocks for synchronization and fine-tuning. Damiola et al. [33**] and Stokkan et al. [35**] showed that restricted feeding uncouples the peripheral clocks from that ticking in the SCN. Because mice are nocturnal animals, they prefer to eat during the night, a behavior presumably regulated by the clock residing in the SCN. When expression of canonical clock genes is measured e.g. in liver of day-time-fed animals, transcript levels peak up to 12 hours earlier than in night-time-fed mice without affecting clock gene expression in the SCN. For example, the level of Per1 mRNA is maximal ~18:00 in the case of nocturnal feeding but achieves its zenith at 06:00 in the case of day-time feeding [33**]. Food-based clock resetting requires several days indicating a clock-based mechanism. Of note, day-time restricted feeding specifically affects the expression of clock genes in the liver but does not result in a global change in liver gene transcription [33**].

What controls the resetting of clock gene expression by restricted feeding? One possibility is that nocturnal body temperature depression resulting from day-time feeding could act as a cue [36*]. The body temperature of mammals cycles with a circadian period and an amplitude of ~3°C but in the case of day-time restricted feeding such fluctuations nearly double [36*]. Further evidence for a critical role of body temperature fluctuation comes from experiments in which naturally occurring circadian temperature
cycle regimens are imposed on cultured fibroblasts and clock gene expression is monitored [36]. Normally, the circadian expression patterns of clock genes of cultured fibroblasts dampens rapidly if cells are kept at constant temperature. By contrast, if rhythmic temperature profiles mimicking natural body temperature curves are applied to culture dishes, the cells contained therein exhibit prolonged and rhythmic clock gene expression. Taken together, restricted feeding studies and rhythmic temperature profile experiments have led to a model in which the SCN controls sleep–wake cycle, locomotor activity, and feeding behavior. The metabolic hormones whose secretion is evoked by feeding in combination with the environment/body temperature rhythms would then coordinate control the circadian pacemaker of peripheral organs [36]. In other words, the central pacemaker indirectly controls clocks in the peripheral organs. It will be important to identify signaling factors and pathways that transmit temperature and hormone signals to the clock proteins driving the peripheral oscillators. One possibility is that candidate SCN output factors such as PK2 and TGF-α also act on peripheral clockworks. A fascinating alternative mechanism by which food intake could entrain the circadian clock has been investigated by McKnight and co-workers ([37]; reviewed in [8]). It has been shown that the redox state of a cell strongly influences binding of CLO CK/BMAL1 and NPAS2/BMAL1 heterodimers to their cognate E-box. Both heterodimers effectively bind to DNA in the presence of reduced NADH and NADPH. By contrast, the presence of oxidized cofactors (NAD⁺ and NADP⁺) is inhibitory. Small changes in the ratio of reduced to oxidized cofactor cause sharp transitions from almost undetectable to near maximal DNA binding. Thus the redox state of a cell that in turn is dependent for instance on food intake may directly affect transcription of Per and Cry genes.

The circadian transcriptome

As in many other areas of biomedical research, global gene expression profiling (e.g. using microarrays) has been applied to the circadian system. Such analyses included the SCN [38*], liver [38*,39*,40–42], heart [39*] as well as synchronized tissue culture cells onto which a circadian rhythm was imposed [43,44]. These studies are a rich source of genes that play a role in all aspects of circadian physiology in the SCN and in peripheral clocks. Several general points have consistently emerged from these investigations. In tissues, ~6–8% of all genes exhibit a circadian expression pattern. These genes associate with diverse biochemical functions and circadian control seems to be most obvious in the rate-limiting step of pathways. Somewhat unexpectedly, there is only a minor overlap of cycling transcripts between tissues. SCN and liver share only 28 genes that are expressed in a circadian pattern, albeit each tissue harbors ~330 cycling genes [38**]. Likewise, heart and liver share only 52 genes [39**]. When genome-wide expression profiling is applied to cycling fibroblasts, the fraction of rhythmically expressed genes is ~2%, significantly less than seen with tissues collected from animals [43,44]. This indicates that it is well worth the effort to use animal tissue as a source of mRNA for expression profiling.

Conclusions

In the course of the past few years, many substantial advances have been made in understanding the biology of the circadian clock. We have now a reasonably comprehensive model of the clock core mechanism but still lack a clear picture of the signaling pathways that transmit environmental cues to the core clock. Light input is at least in part mediated by a photopigment (melanopsin) located in retinal ganglion cells. Non-photic inputs may be hormonal and also may stem from the redox state of cells harboring the clock. There are promising candidates for clock output signals of the SCN such as TGF-α and PK2, but there might be additional possibly endocrine signaling molecules. On the other hand, recent work with food-based entrainment suggests that circadian clocks, prevalent in peripheral tissues (like liver, kidney and heart), may operate quite autonomously. In this case, the master clock of the SCN ‘merely’ acts as a coordinator using straightforward pathways such as locomotion. Nonetheless, the plethora of circadian-regulated genes that have emerged from recent genome-wide expression profiling studies may still yield gene products that function as hormonal output signals. It is needless to say that systematically sorting through the hundreds or perhaps thousands of genes expressed in a circadian fashion is an important effort. Although clock genes regulate the circadian oscillator, they may have additional functions. Per 2, for example, has emerged as a tumor suppressor [45**]. An important field for future research concerns the extrapolation of results obtained with animal models to humans. People differ in the way they stumble into a new day to cope with the tasks they anticipate [46]. To a significant extent, the circadian clock is nature’s tool to effectively deal with the daily routine.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- **of outstanding interest

1. Cho K: Chronic ‘jet lag’ produces temporal lobe atrophy and spatial cognitive deficits. Nat Neurosci 2001, 4:567-568. MRI analysis of the brain of flight attendants exposed to frequent and prolonged transmeridian flights shows temporal lobe atrophy and reduced cognitive performance. This effect may be mediated by elevated cortisol. Effect may be alleviated by appropriate scheduling of recovery periods between flight.


An exogenously defined temperature cycle imposed on cultured cells can establish circadian gene expression in cultured fibroblasts and sustain this circadian rhythm for a prolonged period of time.


The redox state of cells determines affinity of CLOCK protein to DNA. This raises the possibility that such physiologic parameters are important determinants of circadian clock entrainment.


A comprehensive study of genome-wide expression profiling using microarrays. A very rich source of genes expressed in a circadian pattern in the SCN and in liver. Results indicate that nearly 10% of all genes are expressed in a circadian pattern.


Similar to [38] but comparing liver and heart.


Examining Per2 mutant mice, the authors find that PER2 functions as a tumor suppressor and thus this work establishes a connection between the circadian clock and the cell cycle.