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The Mammalian Circadian Timing System: Organization and Coordination of Central and Peripheral Clocks

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Key Words

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Abstract

Most physiology and behavior of mammalian organisms follow daily oscillations. These rhythmic processes are governed by environmental cues (e.g., fluctuations in light intensity and temperature), an internal circadian timing system, and the interaction between this timekeeping system and environmental signals. In mammals, the circadian timekeeping system has a complex architecture, composed of a central pacemaker in the brain's suprachiasmatic nuclei (SCN) and subsidiary clocks in nearly every body cell. The central clock is synchronized to geophysical time mainly via photic cues perceived by the retina and transmitted by electrical signals to SCN neurons. In turn, the SCN influences circadian physiology and behavior via neuronal and humoral cues and via the synchronization of local oscillators that are operative in the cells of most organs and tissues. Thus, some of the SCN output pathways serve as input pathways for peripheral tissues. Here we discuss knowledge acquired during the past few years on the complex structure and function of the mammalian circadian timing system.

INTRODUCTION

SCN: suprachiasmatic nucleus

Most light-sensitive organisms from cyanobacteria to humans are equipped with time-measuring devices, known as circadian clocks, that allow them to anticipate daytime and hence to organize their physiology and behavior in a proactive rather than a responsive manner. As insinuated by their name, circadian clocks ("circa diem" means "approximately a day") cannot measure 24 h with high precision but have to be periodically synchronized to geophysical time. The photoperiod is the most dominant environmental *Zeitgeber* (time giver) for the phase entrainment of circadian oscillators in all investigated organisms, including cyanobacteria, fungi, green plants, and metazoans. In mammals, the circadian timing system is composed of virtually as many clocks as there are cells, as most cells harbor self-sustained and cell-autonomous circadian oscillators. This raises the question of how light can establish phase coherence in cells of opaque organisms.

The answer lies in the hierarchical architecture of the mammalian timing system. Specialized suprachiasmatic nuclei (SCN) neurons receive photic information from the retina via synaptic transmission by axons of the retinohypothalamic tract. This electrical information is converted into chemical information that alters the phase of clock gene expression in a subset of SCN neurons. Because the oscillators of SCN neurons are tightly coupled (see Reference 1), the new phase is rapidly established in all SCN neurons. Owing to the paracrine and synaptic communication of SCN cells, the SCN oscillators never desynchronize in animals deprived of external timing cues. Moreover, the coupled SCN neurons maintain phase coherence during days to weeks in organotypic tissue explants (see Reference 1). The SCN then transmits its rhythmic information to cells in other brain regions and peripheral organs via a variety of outputs. These include neuronal connections, endocrine signals, body temperature rhythms, and indirect cues, provoked by oscillating behavior. For example, rest-activity cycles generate feeding-fasting rhythms, which appear to be dominant *Zeitgebers* for many peripheral organs, such as liver, pancreas, kidney, heart, and skeletal muscles. Although the molecular pathways through which feeding rhythms synchronize peripheral clocks are still poorly understood, it is tempting to speculate that nutrient-sensing hormones or intracellular metabolites may be involved. As revealed by studies on laboratory rodents and cultured cells, many parallel signaling pathways can reset the phase in peripheral cell types, and this redundancy renders the molecular dissection of these synchronization pathways particularly daunting. In this review, we discuss various routes by which the SCN may coordinate circadian physiology in the brain and peripheral tissues.

CENTRAL CLOCKS AND THE BRAIN

As mentioned above, the circadian system of mammals encompasses all organs, tissues, and cells. One of the hallmarks of this system is its ability to synchronize the individual circadian clocks at all levels. The brain, however, has a somewhat special position because it is separated by the blood-brain barrier from the rest of the body. As a consequence, synchronization mechanisms that coordinate peripheral organs and tissues do not necessarily have the same effect on the brain. Only nervous signals and blood-borne lipophilic signaling molecules such as glucocorticoids, which can pass through the blood-brain barrier, can affect the brain.

Synchronization between central and peripheral clocks and synchronization of cellular clocks within the brain impact circadian timing, physiology, and behavior. In the following sections we discuss different oscillators in the brain and their function.

Suprachiasmatic Nuclei: Master Clock or Master Synchronizer?

Initially, circadian rhythms were seen as a diffuse time-measuring capacity of the organism as a whole, until Pittendrigh (2) developed the

idea of a distinct light-sensitive oscillator that serves as a pacemaker for the organism. From then on researchers were investigating brain structures that could serve as pacemakers, mainly using wheel-running behavior of laboratory rodents as a readout. Lesion experiments in the brain led to the identification of a paired structure in the hypothalamus located just above the optic chiasma, the SCN (**Figure 1**). This structure appeared to be important for rhythmicity in corticosterone secretion and locomotor activity (3, 4). Transplantation of fetal SCN tissue into the third ventricle of previously SCN-lesioned hamsters restored circadian locomotor activity (5). Furthermore, SCN tissue from *Tau* mutant hamsters displaying circadian rhythmicity with a shortened period length restored rhythms in SCN-lesioned wild-type hamsters with a period length characteristic of the mutant donor (6). Similarly, SCN grafts from wild-type mice could restore

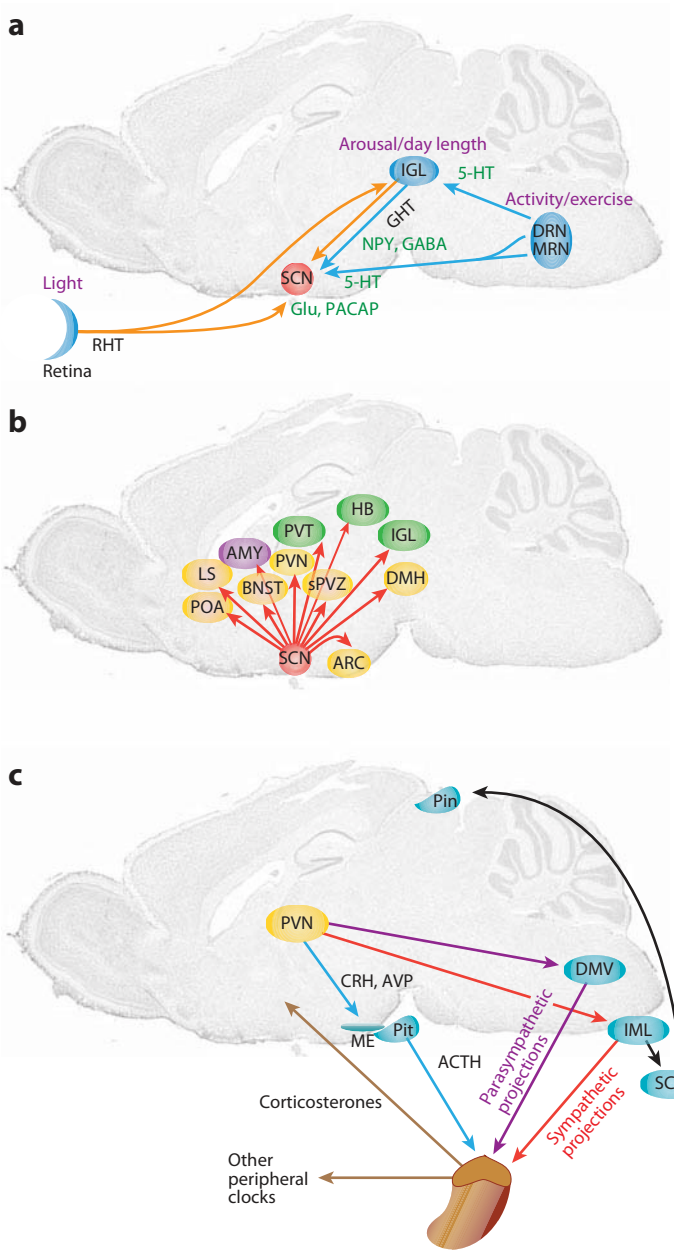


Figure 1

(*a*) Main afferent pathways to the SCN in rat. Orange arrows represent photic input, and blue arrows represent nonphotic input to the SCN. 5-HT, serotonin; DRN, dorsal raphe nucleus; IGL, intergeniculate leaflet; GABA, gamma-aminobutyric acid; GHT, geniculohypothalamic tract; Glu, glutamate; MRN, median raphe nucleus; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase–activating peptide; RHT, retinohypothalamic tract; SCN, suprachiasmatic nuclei. (*b*) Efferent pathways from the SCN (*red*) to hypothalamic (*yellow*) and thalamic (*green*) brain regions. AMY, amygdala; ARC, arcuate nucleus; BNST, bed nucleus of the stria terminalis; DMH, dorsomedial hypothalamus; HB, habenula; IGL, intergeniculate leaflet; LS, lateral septum; POA, preoptic area; PVN, paraventricular nucleus of the hypothalamus; PVT, paraventricular nucleus of the thalamus; SCN, suprachiasmatic nuclei; sPVZ, subparaventricular zone. (*c*) Pathways from the PVN to the adrenal gland. Three different output systems are highlighted: the neuroendocrine neurons of the PVN-controlling pituitary (Pit) hormones (*blue arrows*), parasympathetically projecting neurons in the PVN that target the dorsal motor nucleus of the vagus (DMV) (*purple arrow*), and sympathetically projecting neurons in the PVN that target the spinal cord preganglionic neurons located in the intermediolateral columns (IML) (*red arrows*). From the IML the pineal gland (Pin) is regulated via the superior cervical ganglia (SCG). The neuronal message sets the sensitivity of the organs for the arrival of hormones. The neuronal and hormonal messages from the organs feed back to the brain. ME, median eminence. ACTH, adrenocorticotropic hormone; CRH, corticotropin-releasing hormone; AVP, arginine vasopressin.

RHT: retinohypothalamic tract

GHT: geniculohypothalamic tract

pRGC: photosensitive retinal ganglion cells

VIP: vasoactive intestinal polypeptide

PACAP: pituitary adenylate cyclase–activating protein

circadian rhythmicity in genetically arrhythmic mice (7). These experiments demonstrated a prominent role of the SCN for circadian rhythmicity. When the SCN were isolated as a hypothalamic "island" (8) or when SCN neurons were cultured in vitro, electrical activity was circadian (9–11), even after three weeks in culture (12). Furthermore, individual neurons showed circadian firing rhythms, demonstrating the cellular nature of circadian rhythms (13). Additionally, metabolic activity and glucose uptake in the SCN were dependent on time (14) as well as on expression of clock genes (15, 16) and proteins (17), illustrating clock activity in the SCN at the physiological and molecular levels.

The above-mentioned observations led to the view that the SCN are containing the clock of an organism and that other rhythms may be simply driven by this master clock in the brain, because secretion of vasopressin, one of the peptides transmitted by the SCN, followed the SCN's pattern of electrical and metabolic activity. However, there was evidence that oxygen consumption in rat liver suspension culture was circadian (18), indicating the presence of self-sustaining clocks in the periphery. This notion has been substantiated, leading to the view that a self-sustained clock not only is a property of the SCN and its neurons but is present in most, if not all, tissues, organs, and cells (see below). Therefore, the SCN is probably best viewed as a conductor of an orchestra of clocks that synchronizes all clocks and integrates information from the periphery to generate coherent systemic rhythms in the organism.

The Suprachiasmatic Nuclei: A Relay to Synchronize Physiology to Environmental Changes

To adapt an organism's physiology to changing environmental parameters, information from outside has to reach body structures that can integrate this information and send appropriate signals to various tissues. The SCN have the capacity to serve as such a relay between the external and internal worlds and the body (**Figure 1**).

Input to the SCN: information from the environment. The SCN can be influenced via three major input pathways: the retinohypothalamic tract (RHT), the geniculohypothalamic tract (GHT), and serotonergic (5HT) input from the dorsal raphe nucleus (DRN) and median raphe nucleus (MRN) (**Figure 1*a***). From these three input pathways the RHT mediates photic information (**Figure 1*a***), whereas the GHT and the raphe nuclei provide nonphotic information to the SCN (**Figure 1*a***). The SCN vary in their temporal responsiveness to these stimuli. For example, in nocturnal rodents, exposure to light causes shifts in the phase of the SCN clock primarily during subjective night (the activity period in rodents), whereas nonphotic cues elicit these shifts mainly during the subjective day (the resting period in rodents).

Mammals perceive light information mainly via the retina of the eye, where non-image-forming photoreceptors termed photosensitive retinal ganglion cells (pRGC) (19–21), which express the photopigment melanopsin [a homolog of a photoreceptor in amphibian skin (22–24)], send photic information directly to the SCN via the RHT (25). The monosynaptic RHT fibers end directly on neurons in the ventrolateral part of the SCN that express vasoactive intestinal polypeptide (VIP) (26, 27). The molecules implicated in the photic signaling are the excitatory neurotransmitter glutamate (28) and the neuropeptide pituitary adenylate cyclase–activating protein (PACAP) (29). Their release leads to the activation of several signaling pathways (reviewed in Reference 30) and evokes chromatin remodeling (31) and induction of clock genes (32–34) and immediate early genes (35). One important signaling mechanism for transmitting light information in an SCN neuron involves Ca^{2+} influx and intracellular Ca^{2+} levels modulated by the inositol trisphosphate receptor (36) and the ryanodine receptor (37). Regulation of the ryanodine receptor appears to involve circadian clock proteins in vitro (38), providing evidence that the

clock may regulate components of its own input pathway. Recently, a role of *inhibitor of DNA binding* (Id) genes in the regulation of photic entrainment of the mammalian clock has been proposed (39). In particular, Id2 can inhibit CLOCK:BMAL1 transactivation of *Per1* and *AVP* (arginine-vasopressin) promoters in vitro.

However, the most extensively studied pathway is the extracellular signal–regulated kinase (ERK) pathway. Activation of ERK culminates in the phosphorylation of cAMP response element–binding (CREB) protein (40–42). Phosphorylated CREB binds to cAMP response elements (CRE) located in the promoters of target genes, activating their transcription (43). *Per1* and *Per2* genes contain a CRE within their promoter regions; hence, these core clock genes can be activated independently of the CLOCK:BMAL1-controlled E-box activation (44, 45). Interestingly, these genes are inducible only when light hits the retina during the night (or subjective night, when animals are kept in constant darkness), which is during the time when mammals respond behaviorally with an advance or a delay of periodic activity. This adaptation is termed resetting and probably reflects the necessity of daily adaptation of the internal clock to the current solar time. *Per1* gene induction appears to play an important role in the resetting process because antisense oligonucleotides against *Per1* inhibit phase delays (46) and phase advances (47). Similarly, photic induction of the *Per2* gene may be involved in phase delays (48). Consistent with these observations is the finding that light-dependent phase advances or delays are impaired in mice mutant in the *Per1* or *Per2* genes, respectively (49). However, it appears that behavioral responses to light are strongly dependent on the light history that an organism experienced (50, 51).

Interestingly, the RHT projects not only to the SCN but also to the intergeniculate leaflets (IGL) (**Figure 1*a***) (52, 53). From the IGL the GHT projects to the SCN and therefore can indirectly confer processed light information by releasing neuropeptide Y (NPY) and gamma-aminobutyric acid (GABA) (52, 54). Hence, the SCN receive two different signals upon light stimulation of the retina, one directly via the RHT and the other indirectly via the GHT. The delay between the RHT and the GHT signals may provide additional information leading to a more differentiated response of the SCN to light. This may partially explain the importance of light history in molecular and behavioral responses. Consistent with this view are the observations that the IGL modulates photoperiod responsiveness in Siberian hamsters (55) and that NPY-deficient mice show altered circadian response to simulated "natural" photoperiods (56). The IGL, however, is stimulated not only via the RHT from the retina but also via a nonphotic pathway emanating from the DRN (57) (**Figure 1*a***). This indicates that the pathway via the IGL allows an integration of photic and nonphotic signals to entrain the SCN.

The third important afferent input to the SCN comes from the MRN in hamsters (57, 58) and from both the MRN and DRN in rats (59) (**Figure 1*a***). These fibers end within the VIP-immunoreactive cells of the SCN core region (60, 61), to which the retinal afferents also project (62). When this 5HT path is influenced by administration of agonists or antagonists of 5HT, locomotor activity in both light-dark (LD) and dark-dark (DD) is affected (63). Therefore, it is believed that the 5HT tract participates in nonphotic regulation of the SCN and entrainment of the circadian clock.

Output from the SCN: passing information to the body. To function as a pacemaker and synchronizer for other brain and peripheral clocks, the intrinsic timekeeping signal from the SCN has to be transmitted. In the brain, the connections of the SCN with other brain structures have been elucidated by injecting antero- and retrograde tracers showing that the SCN efferents terminate in a range of brain sites (**Figure 1*b***) (64). Within the hypothalamus SCN efferents terminate most densely in the subparaventricular zone (sPVZ) (65) and rostrally in the preoptic area (POA), the bed nucleus of the stria terminalis (BNST), and the

CREB protein: calcium/cAMP response element–binding protein

LD: light-dark

DD: dark-dark

DMH: dorsomedial hypothalamus

ARC: arcuate nucleus

PVN: paraventricular nucleus

HB: habenula

lateral septum (LS). Dorsally, they terminate in the dorsomedial hypothalamus (DMH) and the arcuate nucleus (ARC). In the thalamus, axons from the SCN innervate the paraventricular nucleus (PVN) and possibly the IGL. Other SCN projections like those to the habenula (HB) and amygdala (AMY) have to be confirmed. Of note, specific subdivisions within the SCN project to designated areas, and these patterns of projections can differ between species (reviewed in References 66 and 67).

Neurochemicals transmit SCN information via the axonal connections mentioned above. Ultrastructural studies have shown that ~30% of SCN axons contain both GABA and peptide neurotransmitters (68, 69). Electrophysiological studies have also shown that glutamate is an SCN transmitter (70) and conveys circadian signals to hypothalamic target structures (71). Additionally, the following molecules have been described as SCN output signals: AVP (72), cardiolipin-like cytokine (73), prokineticin 2 (PK2) (74), VIP (75), and transforming growth factor α (TGFα) (76, 77). For some of these neurochemicals, rhythms of synthesis in the SCN have been observed. However, it is unclear whether mRNA rhythms result in cyclical release of these transmitters in the target sites of SCN efferents.

The best studied of these candidate outputs is PK2. In the SCN its expression peaks in the middle of subjective day, and this pattern of expression is altered by light exposure (78). PK2 receptor mRNA expression is observed in many brain areas known to receive SCN efferents including the DMH, the LS, and the PVN (**Figure 1*b***). Exogenous application of PK2 suppresses locomotor activity in rats during the night but increases it during the day, indicating that PK2 transmits circadian activity of the SCN (74, 79). However, mice lacking PK2 or the PK2 receptor display dampened rhythms in activity and thermoregulation, suggesting that factors other than PK2 can sustain circadian locomotor activity (80, 81).

The transplantation experiments of SCN tissue described above make evident that projections from SCN neurons are not required for the establishment of locomotor activity rhythms. Transplanted SCN tissue can be implanted into many brain areas and restore rhythms, as can fetal SCN grafts encapsulated in material preventing neurite outgrowth into the host brain (82). Thus, it appears that an as-yet-unknown paracrine factor(s) released from SCN tissue can act to coordinate the expression of rhythms in wheel-running activity. The site of action of these factors is unknown, but lesion experiments suggest that the sPVZ is involved. Interestingly, SCN grafts do not restore rhythms in the neuroendocrine axis. This implicates that SCN efferents control hormone rhythms such as melatonin and corticosterone (5, 83).

Hence, the autonomic nervous system plays an important role as a hand of the SCN master pacemaker. In support of this view are data suggesting that the SCN controls (indirectly; see **Figure 1*c***) pineal as well as adrenal cortex function. Tracing studies and physiological experiments indicate that, apart from the classical neuroendocrine control of the adrenal cortex by the PVN-CRH-ACTH (paraventricular nucleus–adrenocorticotropic hormone–corticotropin-releasing hormone) cascade, the autonomic projections of the SCN via the PVN to the IML determine daily changes in sensitivity of the adrenal gland to ACTH (71, 84, 85). This is further highlighted by the finding that light activates the murine adrenal gland, as observed via timing of gene expression and glucocorticoid release (86). Moreover, light affects gene expression via the autonomic nervous system in the liver (87). It appears that the SCN affect not only hormone secretion but also modulate the sensitivity of the target organs of these hormones by neuronal mechanisms. This notion is supported by the finding that injection of transneuronal tracers into various organs/tissues results in the labeling of neurons in the SCN via sympathetic and parasympathetic branches of the autonomic nervous system (88–91). Because neurons in the SCN are labeled via both the parasympathetic and the sympathetic systems, the SCN probably influences both the rest and the activity periods of

circadian rhythms. Taken together, the data indicate that the SCN can transmit circadian information to influence cyclic physiology in peripheral organs not only by hormones but also by direct nervous control of these organs (reviewed in Reference 92).

The findings described above have to be taken with a grain of salt. Although most mammals show circadian rest/activity patterns, some are nocturnal and others diurnal. Hence, hormonal signaling as well as neuronal projections may differ between such organisms. However, in the SCN there is no distinction between nocturnal and diurnal animals with regard to the phase of clock gene expression because the light-dependent synchronization is similar in all investigated species (93, 94). Examination of the mRNA profile of the putative output factor TGFα in the SCN showed that in the diurnal rodent *Arvicanthis ansorgei* the pattern of expression does not match the behavioral rhythm, whereas it does in nocturnal animals (95). Furthermore, the phase of circadian gene expression varies greatly in peripheral tissues of diurnal and nocturnal animals (96). Therefore, the signals emanating from the SCN must be interpreted in an opposite manner in diurnal compared with nocturnal mammals. One plausible explanation for these observations is that the downstream actions of SCN output factors differ between nocturnal and diurnal animals. Identification of such factors will be of great interest to the field of chronobiology.

Multiple Brain Clocks: Multiple Clock Mechanisms?

The first autonomous oscillator in mammals outside the SCN was identified in the retina (97). Cultured retina displayed circadian rhythms in melatonin synthesis that entrained to light in vitro, continued oscillating in constant darkness, and were temperature compensated, all hallmarks of an autonomous oscillator. Cultured retina from *Tau* mutant hamsters showed an accelerated circadian melatonin rhythm of 20 h, which paralleled the period of locomotor rhythms in these hamsters (97, 98), indicating that the oscillations in the retina are generated by similar mechanisms as in the SCN. Interestingly, reports on clock gene expression in the retina are contradictory with regard to the pattern and phase of oscillating gene expression. This hints to variations in the clock mechanism that may depend on tissue- or species-specific factors. For example, a variation in the clock mechanism is observed in the forebrain of mice, where NPAS2 acts as a functional substitute for CLOCK (99, 100). As revealed by loss-of-function genetics, NPAS2 and CLOCK can functionally replace each other in the SCN. In contrast, CLOCK is indispensable for circadian gene expression in peripheral tissues (101).

The discovery of clock genes enabled the identification of brain areas that contain the molecular machinery necessary for the generation of circadian rhythms. Thus, daily oscillations in gene expression have been identified in a number of brain regions (102), including nuclei in the thalamus and hypothalamus, AMY, olfactory bulbs (OB), and cerebellum (see **Figure 2**) (for a comprehensive list, see Reference 103). Transgenic rats expressing the luciferase gene under the murine *Per1* promoter allowed visualization of intrinsic rhythmicity of *Per1* promoter activation in isolated brain regions (104). Twenty-seven different brain regions were cultured, and approximately half were rhythmic. The SCN displayed sustained circadian *Per1* bioluminescence, whereas all other brain regions investigated showed dampened oscillations or were arrhythmic. This finding is in keeping with the observations that the cellular oscillators are coupled in the SCN but not in other brain regions (or peripheral tissues; see Reference 1). From the extra-SCN oscillators the OB (**Figure 2**) showed the most robust rhythms, and tissues with a neuroendocrine function such as the ARC, PVN, and pituitary gland (Pit) displayed only slight dampening of bioluminescence over time. Maximal *Per1* promoter activity was observed during the day in the SCN, but not in other brain regions. Thus, notable differences in phase and robustness as well as in the kinetics of light-induced

OB: olfactory bulbs

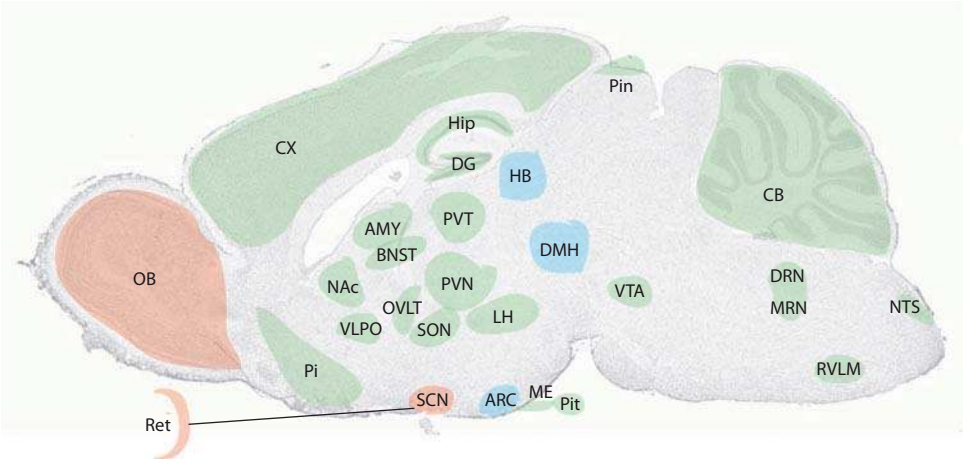


Figure 2

Potential circadian oscillators in the mammalian brain. Self-sustained circadian oscillators are shown shaded in red, semiautonomous oscillators in blue, and slave oscillators in green. AMY, amygdala; ARC, arcuate nucleus; BNST, bed nucleus of the stria terminalis; CB, cerebellum; CX, cortex; DG, dentate gyrus; DMH, dorsomedial hypothalamus; DRN, dorsal raphe nucleus; HB, habenula; Hip, hippocampus; LH, lateral hypothalamus; ME, median eminence; MRN, median raphe nucleus; NAc, nucleus accumbens; NTS, nucleus of the solitary tract; OB, olfactory bulb; OVLT, vascular organ of the lamina terminalis; Pi, piriform cortex; Pin, pineal gland; Pit, pituitary gland; PVN, paraventricular nucleus of the hypothalamus; PVT, paraventricular nucleus of the thalamus; Ret, retina; RVLM, rostral ventrolateral medulla; SCN, suprachiasmatic nuclei; SON, supraoptic nucleus; VLPO, ventrolateral preoptic area; VTA, ventral tegmental area.

phase resetting exist between different brain areas (104, 105). Attenuated oscillations in extra-SCN brain rhythms may be attributable to either a lower robustness of oscillators in individual cells or a less efficient synchronization of individually oscillating cells. Fibroblasts kept in culture display robust oscillations, but because individual cells have slightly different periods, they desynchronize over time. Hence, after a transient synchronization by a serum shock or a dexamethasone treatment, oscillations of gene expression in the entire population dampen after a few days (106, 107). Similarly, in cultured OB, individual neurons display circadian oscillations in *Per1*:luciferase expression that differ in phase and periods, and therefore the compound rhythms of OB are lost over time (108). In the intact animal, signals from the SCN appear to synchronize populations of weakly coupled or noncoupled cells in the brain, rather than impose rhythmicity on otherwise arrhythmic cells in non-SCN brain regions.

The brain is composed of various cell types; neurons and glia are the most prominent ones. Neurons and glia are metabolically dependent on each other (109) and have complementary functions. The degree of metabolic dependency as well as the electophysiological communication between these two cell types may vary in different brain regions, which may have consequences on the degree of synchronization between these cells. Therefore, it is important to evaluate whether the circadian clock mechanism as well as the synchronization properties of these two cell types in the brain are identical. A recent study evaluated the expression of the clock genes *Per1* and *Per2* using mice expressing the Venus and DsRED proteins under the control of the *Per1* and *Per2* promoters, respectively (110). Consistent with previous observations (102), the expression of the *Per1* and *Per2* transgenes was only partially overlapping in various brain regions. Detailed cellular analysis in the dentate gyrus (DG), striatum, and

cortex revealed that *Per2*:DsDRED is mainly observed in cells expressing glial fibrillary acidic protein (GFAP), a marker for glial cells. In contrast, *Per1*:Venus is expressed in neurons and to some extent also in glial cells (110). These results indicate cell type–specific segregation of *Per1* and *Per2* promoter activation, suggesting a possible divergent role of *Per1* and *Per2* in mouse brain, as suggested previously on the basis of microarray data (111). Furthermore, these results indicate that variations in the clock mechanism in neurons and glia may exist. In line with this view is the observation that *Per2* mutant mice have a specific defect in astrocytes (a glial cell type). In these animals the astrocyte-specific glutamate transporter Eaat1 (Glast) is reduced in its expression, and as a consequence uptake of glutamate by astrocytes is reduced, leading to an inefficient clearance of glutamate from the synaptic cleft (112). Future experiments will hopefully shed light on the importance of the segregation of *Per1* and *Per2* gene activation in the two main cell types in the brain and the impact such segregation may have on intercellular coupling and cell-autonomous oscillations.

In the brain, the functional relevance of circadian clock gene expression can be measured by evaluating the firing rate of neurons. In the SCN, similar periods of clock gene expression and firing rates are observed. In particular, *Per1* transcription positively correlates with firing rate in a linear fashion in *Per1*:GFP (green fluorescent protein) mice (113). In vasoactive intestinal peptide receptor 2 (VPAC2) receptor–deficient mice, *Per1*, *Per2*, and *Cry1* gene expression is attenuated in the SCN (114), and similar blunted rhythms are observed in the cellular excitability in these animals (115–117). However, it is unclear whether all extra-SCN brain regions translate clock gene expression cycles into physiologically relevant firing rate rhythms. Cell firing activities outside the SCN were evaluated in several studies (118), but only a few of the brain areas, including the OB and the lateral HB, maintained electrical rhythms and oscillating clock gene expression in the absence of the SCN (105, 108, 119).

An independent self-sustained circadian oscillator in the OB may be important to regulate the sensitivity level of olfactory neurons, thus allowing an animal to follow an odor concentration gradient toward a food source in a daytime-dependent manner. Likewise, increased responsiveness to stimulatory signals from potential mates may be advantageous. The HB has been proposed to contain at least a semiautonomous oscillator (119). Because the HB receives direct nonvisual projections from the retina (120), and because together with the nucleus accumbens (NAc) (121) the HB projects to the pineal gland, the raphe nuclei, the substantia nigra, and the ventral tegmental area (VTA) (122–125), the HB may be important in the integration of diurnal signals to regulate reward-related behavior. SCN-independent oscillators in particular brain regions may help an animal to temporally coordinate its basic needs, such as feeding and mating.

Brain Clocks, Drugs, and the Reward System

Brain structures that regulate and control behavior by inducing pleasurable effects make up the reward system. These structures include, among others, the VTA, the NAc, the HB, and the prefrontal cortex. The presentation of a reward more than once reinforces the intensity of a given behavior. Primary rewards are elicited by food, water, and sex, which are all necessary for survival of the individual and/or species. Secondary rewards derive their values from primary rewards and, for humans, include music, pleasant touch, and money. Rewards modify behavior, modulate learning capabilities, and influence mood. For example, food, alcohol, and cocaine positively influence the reward system, improve subjective well-being, and encourage repetitive ingestion, which will eventually lead to a compulsive behavior. Such reward-related behavior exhibits a recurring pattern with a period of approximately 24 h (126–130), indicating an interaction between the circadian and the reward systems in the brain. Seasonal patterns also appear to influence the use of

addictive drugs such as alcohol, which is often accompanied by depression (131). This seasonal incidence of affective illness is reflected in biochemical determinants (132), suggesting a cellular and molecular basis of such disorders. In support of this view are observations that humans with genetically determined sleep disorders are more prone to addiction (133). Rats with an abnormal circadian rhythm and mice with a mutation in the *Per2* gene absorb ethanol at increased levels when simultaneously offered pure drinking water and a dilute solution of alcohol (112, 134).

Further evidence for the involvement of clock components in drug-induced behavior comes from mice with disrupted *Per1* or *Per2* genes. In these animals locomotor sensitization and conditioned preference for cocaine are abnormal (126). Furthermore, expression of these genes is induced by cocaine in the dorsal striatum and the NAc, brain regions important for cocaine-mediated behavioral effects (135, 136). Interestingly, cocaine differentially affects expression of clock genes in the brain, depending on the treatment schedule (acute or chronic) and the brain area (137). Cocaine and other drugs of abuse such as methamphetamine (see below) influence the reward system in part by modulating dopamine neurotransmission in the mesolimbic dopamine reward circuit, including the VTA and NAc of the striatum (138, 139) (**Figure 3**). Several interactions between dopamine and the circadian clock have been reported. Dopamine neurons in the retina regulate adaptation to light (140), and dopamine D2 receptor–null mice show impaired light masking (141). Signaling via the dopamine D2 receptor also potentiates circadian transcriptional regulation in the retina (142). 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 (143). Furthermore, these animals display a mania-like phenotype similar to that observed in patients with bipolar disorder (144). A molecular link between the circadian clock mechanism and dopamine metabolism has just recently been established (145). In mice, the clock proteins BMAL1, NPAS2, and PER2 regulate expression levels and activity of monoamine oxidase A (MaoA), an enzyme important in dopamine degradation. In this mechanism, PER2 acts as a coactivator and modulates dopamine levels in the mesolimbic dopaminergic system. This has an impact on mood-related behavior in mice, leading to the conclusion that the clock can influence mood. In support of this conclusion is that seasonal affective disorder (SAD) in humans correlates with the frequency of specific single-nucleotide polymorphisms in *BMAL1*, *NPAS2*, and *PER2* (146).

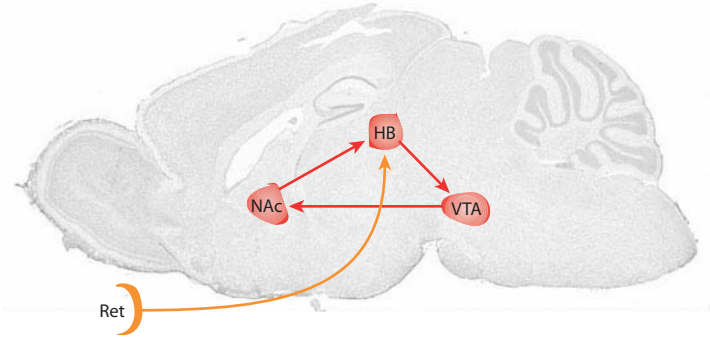


Figure 3

A potential role of the habenula (HB) in the reward system. The ventral tegmental area (VTA)–nucleus accumbens (NAc) pathway plays a critical role in reward. Dopaminergic projections innervate the NAc. How much stimulation from the VTA reaches the NAc may be influenced by the HB, which signals to the VTA when no reward is expected. The NAc in turn feeds back to the HB, thereby establishing a regulatory circuit balancing dopamine levels in the brain. Direct light input to the HB may influence dopamine balance and adjust it to the diurnal cycle. Ret, retina.

Methamphetamine-sensitive circadian oscillator (MASCO). The influence of drugs of abuse on circadian behavior was first studied in rats (147) by using methamphetamine [(2*S*)-*N*-methyl-1-phenylpropan-2-amine]. Methamphetamine affects locomotor activity, the length of alpha (the duration between onset and offset of daily activity), and the period length of the activity rhythm. Even in constant darkness, these effects persist but disappear after withdrawal of methamphetamine. Interestingly, methamphetamine can induce

robust activity rhythms in SCN-lesioned rats (148) as well as in *Clock* and *Cry1/Cry2* mutant mice that are arrhythmic under constant darkness conditions (149, 150), suggesting that an oscillator that is independent of the SCN and is sensitive to methamphetamine must exist. All the behavioral responses observed in rats were confirmed in mice, and these effects do not depend on rhythmic consumption of methamphetamine, indicating that a methamphetamine-sensitive circadian oscillator (MASCO) also exists in mice (151). Because methamphetamine acts primarily on dopaminergic cells in the brain, the mesolimbic-dopaminergic system (VTA, NAc, striatum) (see **Figure 3**) is probably affected by this drug. This would lead to alterations in dopamine (or other catecholamine) levels in the brain.

At the molecular level, repeated injections of methamphetamine cause a sensitized increase in *Per1* gene expression specifically in the mouse striatum without affecting *Per1* or *Per2* gene expression in the master clock of the SCN (152). Furthermore, acute injection of methamphetamine increases the expression of *Per1*, *Bmal1*, and *Npas2* genes in the striatum. Additionally, chronic daytime methamphetamine treatment shifts rhythmic *Per1* and *Per2* expression in the striatum from a nocturnal to a diurnal rhythm without affecting the rhythm in the SCN (153). Among genes altered in expression by methamphetamine treatment, the clock gene *Per2* was most affected (154). Interestingly, mutation of the *Per2* gene leads to a reduction of dopamine degradation (155), highlighting the mutual interaction of the circadian clock with dopamine levels in the striatum. This suggests that known clock components either are part of the MASCO or link the MASCO to the regular circadian oscillator (RCO). In favor of the second possibility are the findings that all clock gene mutants having methamphetamine in their drinking water can display rhythmic behavior with a period length of between 25 and 37 h, even when the SCN is surgically removed (156). Therefore, one can hypothesize that some clock components are involved in coupling the long-period MASCO to the shorter-period RCO to bring the two into resonance. The molecular makeup of the MASCO remains to be investigated.

MASCO: methamphetamine-sensitive circadian oscillator

Because it appears that the MASCO is an SCN-independent oscillator, the question arises as to what the anatomical substrate for MASCO might be. A Mn^{2+}-enhanced MRI study revealed neuronal dysfunction of a long-projecting multisynaptic pathway in response to methamphetamine after injection of Mn^{2+} in the HB (157). This pathway includes the VTA, striatum, and NAc, which as discussed above make up the mesolimbic dopaminergic system. Hence, it appears that methamphetamine affects the mesolimbic dopaminergic system (**Figure 3**), in which dopamine degradation is at least in part under circadian clock control (155). But what role does the HB play? A recent investigation shows that neurons in the lateral HB signal to dopamine neurons when no reward is expected (158). This finding is of great importance because the firing rate of dopamine neurons increases when a reward is given unexpectedly and decreases when an expected reward is omitted. This firing has been proposed to reflect reward prediction errors, a parameter that reflects the difference between the expected as opposed to the actually obtained reward value (159). Thus, the lateral HB seems to be critically involved in predicting reward. Because the HB is directly innervated by the retina (120), it has the possibility, at least theoretically, of relating reward with the day/night cycle and thus modulating behavior toward maximal efficiency for obtaining a reward such as food. In a natural setting, this is of utmost importance for an animal to survive in a competitive environment.

Food-entrainable oscillator (FEO). When food availability is limited to a few hours during each day, mammals quickly alter daily rhythms of physiology and behavior, such as locomotor activity, body temperature, and corticosterone secretion, to correlate with the food availability rhythm (160–162). Increases in locomotor activity occur shortly before the time of daily food presentation and is called food-anticipatory

FAA: food-anticipatory activity

FEO: food-entrainable oscillator

TTFL: transcriptional/translational feedback loop

activity (FAA). FAA appears in SCN-lesioned animals, yet only under a 24-h feeding schedule, demonstrating the existence of an SCN-independent food-entrainable oscillator (FEO) that is not mimicked by a passive hourglass mechanism or by an associated memory process (161, 163). The anatomical substrate for the FEO is still unknown. Lesion of candidate areas such as the hypothalamus or limbic system has failed to identify a principal site (164, 165), supporting the possibility that the anatomical substrata for the FEO are dispersed in various brain regions or located in peripheral tissues (166). However, recent experiments suggest that the integrity of the dorsomedial hypothalamus (DMH) is important, as lesions in this region can affect behavioral expression of FAA in rats (22; but see also Reference 167). Furthermore, robust oscillations of *Per1* and *Per2* gene expression are seen in the DMH only under restricted feeding conditions (168). Interestingly, *Per2* but not *Per1* mutant mice exhibit attenuated food anticipation in mice, as monitored by wheel-running activity, spontaneous locomotor activity, and body temperature (169). Mice with a mutation in the *Clock* gene show normal FAA (170), but mutation of its analog *Npas2* delays FAA in mice (99). Similarly, *Cry1/Cry2* double-knockout mice display delayed onset and reduced stability of FAA (171). Mice deficient in the *Bmal1* gene show robust FAA (172, 173). Overall, it appears that FAA does not necessarily require the known clock genes. Blunted FAA in *Per2* mutant animals, containing a *Per2* gene with a small in-frame deletion (111), may be due to an effect on FAA that is independent of the function of *Per2* in the circadian clock. This finding may highlight a critical link between the FEO and the RCO in which *Per2* may have the role of a synchronizer comparable to its role in linking the activating and inhibiting loops of the core oscillator (174).

Similarities between MASCO and FEO. In a natural setting, animals seek food, and the rewarding properties of food strongly influence food-seeking behavior. Similarly, drug addiction relies on the action of compounds like cocaine or methamphetamine to stimulate brain regions that convey pleasure, which provides motivation and promotes drug-seeking behavior. Food and drug-seeking behavior appear to be linked via hormones, which regulate not only feeding but also neuronal activity in the mesolimbic dopaminergic pathway. In particular, leptin reduces the firing rates of dopaminergic neurons (175), indicating that leptin exerts a direct influence on dopamine neurons via leptin receptors in the VTA (176). Interestingly, leptin-deficient mice show reduced levels of dopamine in the NAc, and leptin treatment increases the synthesis and activity of tyrosine hydroxylase (TH) (176). Mice with a mutation in the *Clock* gene display elevated leptin levels (177) and TH activity with increased dopamine levels in the VTA (143). Hence, feeding abnormalities and abnormal behavior of *Clock* mutant mice in mood-related behavioral tests (144) appear to be related. However, it remains to be determined whether the anatomical substrates of the MASCO and FEO are identical or overlap to some degree.

PERIPHERAL CLOCKS

Circadian Oscillators Are Functional in Most Body Cells

Shortly after the discovery of the first bona fide clock genes in mammals, it became obvious that circadian clocks are ticking not only in neurons of SCN but also in most, if not all, peripheral tissues (106, 178). Moreover, peripheral clocks appear to have a similar molecular makeup as the clocks operative in SCN neurons (179). Thus, in both SCN cells and peripheral cells, the rhythm-generating molecular circuitry is thought to be based on a delayed transcriptional/translational feedback loop (TTFL) involving essentially the same core clock components (see Reference 1). Intriguingly, cultured cells (106) and tissue explants from liver, lung, kidney, spleen, pancreas, heart, stomach, skeletal muscle, lung, cornea, thyroid gland, and adrenal gland exhibit robust circadian oscillations in gene expression (178, 180–182). In

contrast, analysis of clock gene expression in mouse thymus and testis revealed somewhat conflicting results: One research group could not find cyclic mRNA accumulation, whereas another described a 12-h cycle for clock gene expression (183, 184). Circadian oscillators may not function properly in the immature and differentiating cells that make up a large proportion of these two tissues. For example, embryonic stem cells, which can be regarded as the least-differentiated cells, do not appear to be capable of circadian rhythm generation (E. Kowalska & S.A. Brown, personal communication).

In peripheral organs a large number of key physiological functions are subject to daily oscillations. These include xenobiotic and endobiotic detoxification by liver, kidney, and small intestine (185); carbohydrate (186) and lipid (187) metabolism by liver, muscle, and adipose tissue; renal plasma flow and urine production; and parameters of the cardiovascular system such as blood pressure and heart beat rates (reviewed in Reference 188). Given the hierarchical architecture of the circadian timing system, these peripheral functions can be coordinated by systemic cues emanating from the SCN, such as neuronal signals and circulating hormones or metabolites, and/or by local peripheral oscillators synchronized by the SCN (for reviews, see References 188–190).

Genome-wide transcriptome profiling studies performed on peripheral tissues such as liver, heart, and adrenal gland suggest that many cellular functions are subject to circadian regulation. Depending on the tissue and on the algorithms used for data mining, between 2% and 10% of all detected genes are rhythmically expressed. The liver is the most extensively studied organ in terms of circadian transcription. Independent studies have revealed close to 1000 circadian transcripts in the liver (191–200).

As expected, many of the rhythmically expressed liver genes encode key enzymes involved in metabolic pathways, energy homeostasis, food processing, and detoxification (185). It could be argued that economizing energy expenditure is the major virtue of temporally limited gene expression. However, cells are generally quite wasteful with their fuel resources (see Reference 201 and references therein), and it is thus unlikely that the minor energy savings associated with the temporally gated activity of a few genes is the major purpose of circadian gene expression. Rather, the sequestration of chemically incompatible processes to different time windows and the temporal limitation of potentially harmful processes to time spans during which they are absolutely required may constitute the major tasks of circadian peripheral clocks (see Reference 202 and references therein). For example, a simultaneously high expression of glycogen synthase and glycogen phosphorylase would not be compatible with the conversion of glucose into glycogen and vice versa during the absorptive and postabsorptive phases, respectively. Hence, the antiphasic expression of these two enzymes in liver makes physiological sense (203). Furthermore, cytochrome p450 mono-oxidases, which are involved in xenobiotic detoxification, can produce genotoxic reactive oxygen species from molecular oxygen and thereby cause collateral damage. Therefore, the tight regulation of such enzymes by both hepatocyte oscillators and acute regulatory mechanisms may be meaningful. We thus hypothesize that the anticipation of metabolic pathways to optimize food processing, the limitation of metabolic processes with adverse side effects to time periods when they are needed, and the sequestration of chemically incompatible reactions to different time windows are the three most important purposes of peripheral clocks, at least in metabolically active tissues (reviewed in Reference 202).

Intriguingly, Hogenesch and colleagues (193) have recently demonstrated that, in addition to mRNAs with a 24-h accumulation cycle, two clusters of transcripts cycling with 12-h and 8-h periods exist in liver. This original discovery further suggests the significance of oscillating gene expression for the temporal separation of metabolic processes.

Studies published during the past few years provide evidence that posttranscriptional mechanisms are also implicated in the fine

tuning of circadian gene expression. Thus, in liver many proteins issued from constitutively accumulating mRNAs oscillate in abundance, supposedly owing to their rhythmic translation and/or degradation rates (204). Moreover, the hepatocyte-specific microRNA *miR122*, whose transcription is under the control of the clock component REV-ERBα, affects the cyclic expression of numerous regulators and enzymes implicated in lipid and cholesterol metabolism (205).

Properties of Peripheral Oscillators

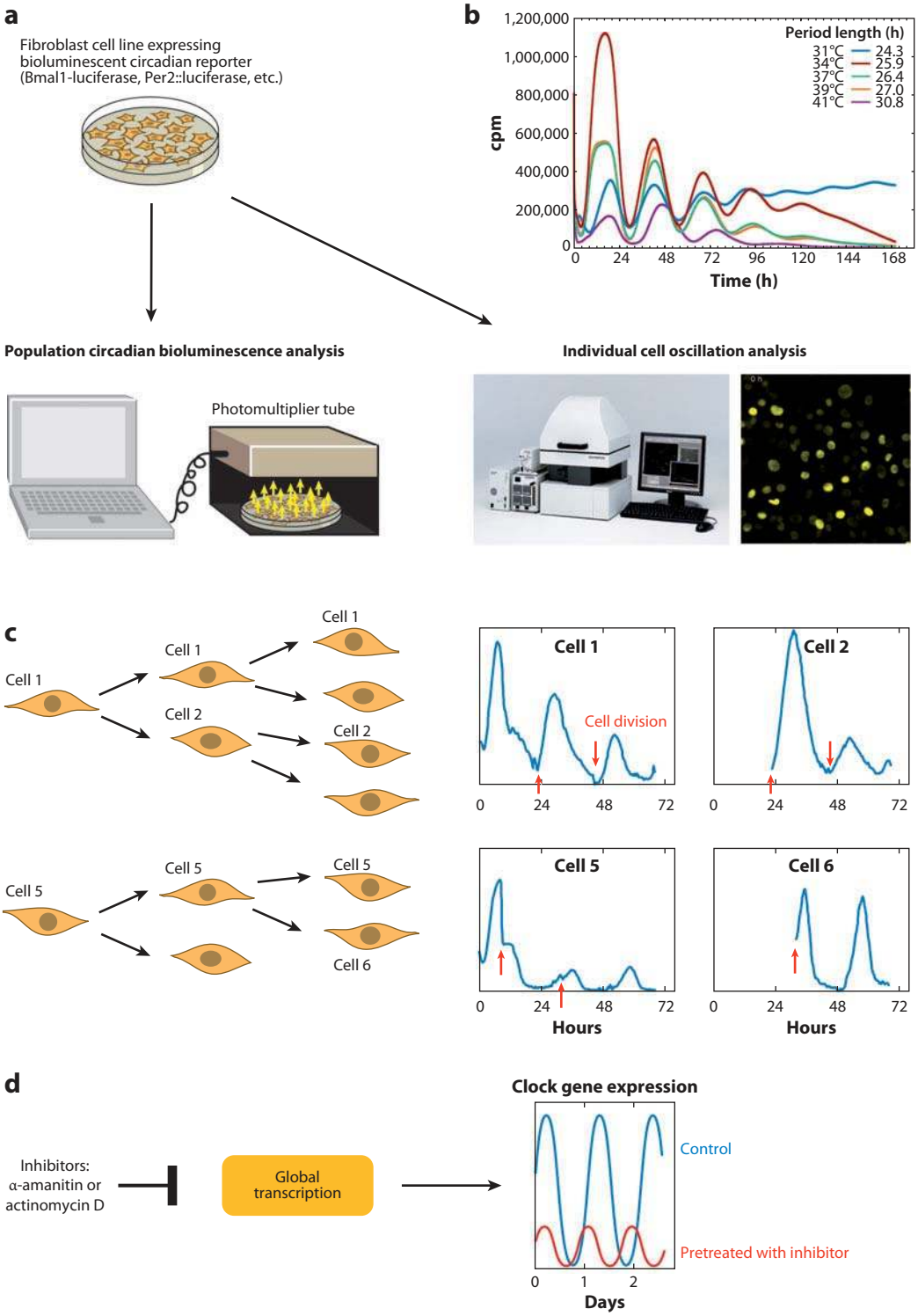
It was originally thought that only oscillators in SCN neurons are self-sustained, whereas the peripheral clocks dampen after several cycles. However, Yoo et al. (182) have shown that circadian timekeepers in liver and lung explants can generate up to 20 (or more) daily cycles of *Per2*-luciferase expression. In spite of the resemblance between central and peripheral clocks, the terms of master and slave oscillators are still justified. In fact, the phases of peripheral oscillators exhibit large differences in SCN-lesioned animals. Furthermore, Bittman and colleagues (206) demonstrated that a functional SCN is required to maintain phase coherence between hepatocytes of the same liver. Hence, peripheral oscillators do not appear to be coupled via paracrine communication signals (206). This latter report also suggests that the transplantation of an SCN into SCN-lesioned animals can restore phase coherence of circadian gene expression in liver, kidney, and skeletal muscle, but not in heart, spleen, or adrenal medulla. Perhaps the synchronization of circadian oscillators in the latter three tissues requires inputs from the peripheral nervous system, which probably cannot be restored by an implanted SCN. In a separate study, Bittman and colleagues (207) also performed elegant parabiosis experiments demonstrating that diffusible SCN-derived signals are sufficient for the phase entrainment of liver and kidney.

In 1998 Balsalobre et al. (106) showed that immortalized Rat-1 fibroblasts, which had been propagated in culture for many decades, possess robust circadian oscillators. Since then, circadian gene expression has been observed in a variety of cultured cell lines and primary fibroblasts, including murine NIH-3T3 fibroblasts (208), mouse and human primary fibroblasts (209), and human U2OS osteosarkoma cells (193). The circadian oscillators of such cultured cells can be transiently synchronized by a puzzling variety of signaling pathways involving both transmembrane and nuclear receptors (188). Single-cell recordings and mathematical analysis of bioluminescence cycles generated by cell populations revealed that fibroblasts harbor self-sustained, cell-autonomous oscillators (107, 210) (**Figure 4**), in keeping with the observations made with peripheral tissue explants (182). The fibroblast clocks exhibit robust but desynchronized oscillations under normal continuous culture conditions. However, as outlined above, these clocks may be transiently synchronized by the activation of several signal transduction pathways.

Peripheral clocks are resilient to large fluctuations in temperature and overall transcription rates. The studies with cultured fibroblasts suggest that, when examined at the single-cell level, peripheral oscillators are at least as robust as those operative in isolated SCN neurons. Thus, oscillators in cultured

Figure 4

Robustness of mammalian cellular clocks. (*a*) Experimental system for the analysis of circadian gene expression in cell populations or single cells (adapted with permission from References 107 and 215). (*b*) Temperature fluctuations within the physiological range do not dramatically alter period length. Surprisingly, the cellular circadian oscillators run slightly faster at lower temperatures (temperature overcompensation). (*c*) During cell division the circadian oscillators keep ticking in daughter cells, and in spite of the sudden division of the cell's contents during this process, the phases undergo only minor advances or delays (adapted from Reference 107). (*d*) Inhibition of RNA synthesis by α-amanitin (an inhibitor of RNA polymerase II–dependent transcription) or actinomycin D (an inhibitor of RNA polymerase I– and II–dependent transcription) does not abolish circadian oscillations.



fibroblasts and SCN neurons keep ticking throughout a cell's lifetime. In fibroblasts, circadian gene expression even persists during cell division, and the phase of these oscillations is passed on to daughter cells with only minor advances or delays. Another remarkable aspect of peripheral clock robustness is temperature compensation, a property described several decades ago for many organisms (211, 212). In contrast to most chemical and biochemical processes, which are accelerated with increasing temperature, the period length of circadian oscillators remains nearly constant over a wide range of physiological temperatures (213, 214). This feature even applies to circadian oscillators operative in cells of homeotherm organisms, such as cultured mammalian fibroblasts. In fact, the oscillators of these cells are even temperature overcompensated, in that they tick slightly faster at lower temperatures (215–217) (**Figure 4*b***). In *Drosophila*, the Per protein plays a key role in temperature compensation, and per^{L} mutant flies lose temperature compensation and exhibit longer periods at higher temperatures (211). We found that *Per1*-deficient primary mouse tail fibroblasts, in contrast to their wild-type counterparts, are not temperature (over)compensated. Rather, they oscillate with similar and even slightly shorter periods at elevated temperatures. This suggests that PER proteins play an important role in the temperature control of circadian oscillators in mammalian cells as well (215).

Besides being temperature compensated, peripheral clocks also appear to be resilient against fluctuations in oscillator components. As mentioned above, cell division, during which the cellular contents are cut approximately in half, does not abrogate oscillator function but causes only relatively modest phase shifts (**Figure 4*c***) (107). Fibroblast clocks also support large variations in general transcription rates. Thus, circadian oscillator function persists in mouse fibroblasts exposed to sublethal doses of the transcription inhibitors α-amanitin and actinomycin D, which lower general RNA polymerase II–dependent transcription by up to threefold (**Figure 4*d***) (215). Breathtaking work from Kondo and colleagues (218) recently revealed that in cyanobacteria circadian protein phosphorylation can persist in the absence of transcription and translation, at least at certain temperatures. Moreover, this group succeeded in reconstituting a circadian oscillator in the test tube with just three clock proteins (KaiA, KaiB, and KaiC) and ATP (219). However, the same research team recently showed that circadian clock gene transcription is required for fully operative clock function in vivo (220). Hence, the coordinated interaction between posttranslational and transcriptional/translational regulatory mechanisms appears to account for the generation of robust daily rhythms in cyanobacteria. Work on mammalian and insect oscillators resulted in similar conclusions for metazoan systems (221, 222), which may explain why absolute cellular concentrations of known clock components do not appear to play preponderant roles in keeping the clocks ticking (see above). The latter notion is also supported by a recent report by Fan and colleagues (223), demonstrating that the overexpression of CRY1, CRY2, and BMAL1, at least within certain limits, does not abrogate oscillator function.

The resilience of cellular clocks to changes in temperature and gene expression may be even more important in peripheral cell types than in the SCN. In contrast to SCN oscillators, whose intercellular communication reinforces their resilience to perturbations (224), peripheral clocks are rather autistic and must thus rely on cell-autonomous robustness. For example, cellular transcription rates can vary dramatically (>20-fold) in different tissues (225). Moreover, even in homeotherm animals like mammals, the ambient temperature can fluctuate significantly between internal organs (e.g., liver, kidney) and tissues exposed to outside temperature (e.g., skin, mucosa, testicles). In addition, fever and hypothermia can lead to large temperature changes, even in internal organs. Because small period-length alterations can result in large phase changes, the robustness of peripheral clocks may be important to assure phase coherence.

An intriguing area of research concerns the balance between different core clock components necessary for proper clockwork function. The single-cell analysis of fibroblasts from *Per1* knockout mice performed by us and others (224) revealed that the low-amplitude oscillations exhibited by these cells at the population level are the result of a small fraction of robustly cycling cells and a large fraction of arrhythmic cells. Therefore, some cells appear to be more sensitive to the absence of PER1 than do other cells, perhaps because PER2 production is upregulated in these cells.

Fibroblast oscillators and behavior. Clock genes are operative in most, if not all, skin cell types and may play a role in several processes such as cellular proliferation (226) and hair follicle cycling (227). Brown and colleagues (209) have compared the wheel-running behavior and the period length of skin fibroblasts of various mice carrying mutations in different core clock genes. Even though the cellular clocks operative in fibroblasts did not perfectly reflect the central clockwork, there was a qualitative correlation between locomotor activity and fibroblast gene expression. Given that the assessment of the circadian period length in human beings is complicated and expensive, the possibility of studying human circadian oscillator properties by using primary fibroblasts was a welcome opportunity. Thus, the skin fibroblast system was also used successfully to characterize amplitude and phase shift properties in individuals with "normal" period lengths but different behavioral phenotypes. These studies suggested that human chronotypes may be influenced not only by the period length of their circadian oscillators but also by cellular components that affect period length, amplitude, and phase (228). Moreover, this system may be exploited for diagnostic purposes, conceivably for subjects with circadian disorders. For example, Vanselow and coworkers (229) have found that a mutation in an *mPer2* phosphoacceptor site corresponding to the *hPER2* mutation associated with human familial advanced sleep phase syndrome (FASPS) phenocopies the short period and advanced phase in cultured fibroblasts.

Synchronization of Peripheral Clocks

As mentioned above, the SCN central pacemaker must establish phase coherence in the body by synchronizing billions of individual cell clocks every day (202, 224). The SCN uses many routes to establish phase coherence in the periphery. Thus, feeding rhythms, driven by rest-activity rhythms, are strong *Zeitgebers* for many tissues (178, 230). Likewise, body temperature rhythms, influenced directly by the SCN and by activity cycles controlled by the SCN, appear to play a role in the resetting of peripheral timekeepers (231). In addition, the SCN also uses more direct timing cues, such as humoral and neuronal signals, to entrain the phases of peripheral clocks (87, 232) (**Figure 5**). Peripheral clocks continue to oscillate in SCN-lesioned mice, but their phases are no longer coordinated in these behaviorally arrhythmic animals (182, 206).

Entrainment of peripheral clocks by indirect cues imposed by oscillating behavior: feeding-fasting cycles and temperature. Daily feeding-fasting cycles appear to be the dominant *Zeitgebers* for several peripheral organs, including liver, kidney, pancreas, and heart. The timing of food intake influences the expression profile of many circadian genes in these organs. Normally, the feeding-fasting cycles are in phase with rest-activity cycles. Strikingly, daytime-restricted feeding of nocturnal rodents inverts the phase of gene expression in peripheral organs, thereby uncoupling peripheral clocks from the SCN (230, 233). The entrainment pathways from feeding-fasting cycles may include hormones secreted upon feeding and fasting [for example, cholecystokinin, peptide YY, oxyntomodulin, ghrelin, leptin (234)], food metabolites (for example, glucose, cholesterol, fatty acids, heme), postprandial temperature elevations, and intracellular redox state [NAD(P)H/NAD(P)$^+$ ratio (235 and references therein)].

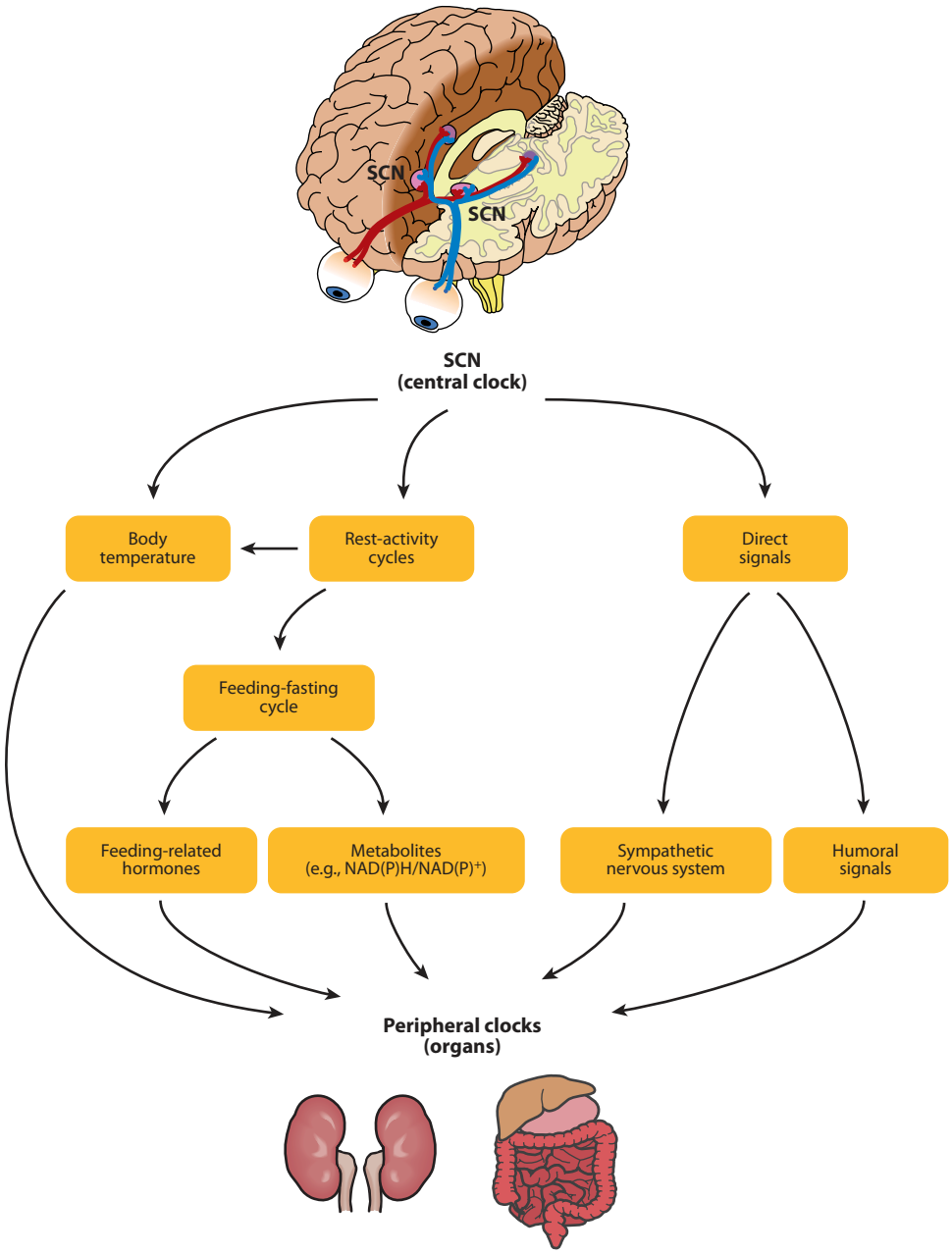


Figure 5

Peripheral clock entrainment pathways (see text for explanations).

There is growing evidence for the interplay between energy metabolism and the circadian clock (236–238). First, the dominance of feeding cycles as a *Zeitgeber* for peripheral clocks implies that the circadian timing system plays an important role in nutrient processing and energy homeostasis. In addition, as we mentioned above, transcriptome profiling

studies revealed that many genes involved in metabolism are rhythmically expressed (191, 192, 194, 199, 239, 240). Furthermore, at least in vitro, the DNA-binding activity of BMAL1-CLOCK is strongly influenced by the ratio of reduced to oxidized NAD cofactors, which are often considered to be readouts of the cellular metabolic state (241).

Mammalian SIRT1, an NAD^+-dependent deacetylase, has been recently identified as a novel regulator of circadian gene expression (242, 243). SIRT1, the mammalian ortholog of yeast Sir2, is involved in transcriptional silencing, genome stability, and longevity (244, 245). Asher and colleagues (242) demonstrated that SIRT1 exhibits circadian accumulation in mouse hepatocytes and cultured fibroblasts and that it is essential for high-magnitude circadian transcription of several core clock genes, including *Bmal1*, *Rorα*, *Per2*, and *Cry1*. Moreover, SIRT1 binds CLOCK-BMAL1 in a circadian manner and promotes the deacetylation and subsequent degradation of PER2 (242).

Temperature fluctuations represent an important entrainment cue in *Drosophila*, *Neurospora*, and mammals (246 and references therein). Shallow temperature rhythms imitating body temperature fluctuations can maintain previously induced rhythms in peripheral oscillators, and can phase shift peripheral clocks, without affecting the phase of SCN (231). In zebrafish kept in constant darkness, temperature steps of as little as 2°C can entrain circadian rhythms in developing larvae (and in zebrafish cell lines) (247).

Using a novel screen dubbed differential display of DNA-binding proteins (DDDP) of mouse liver nuclear extracts, Reinke and coworkers (248) have found a highly rhythmic activity of heat-shock factor 1 (HSF1). HSF1 drives the expression of heat-shock proteins at the onset of the dark phase, when the animals start to be behaviorally active. The onset of feeding is followed by a postprandial rise in body temperature, which makes it difficult to discriminate between temperature-dependent and chemical HSF1 activation pathways. Nevertheless, recent studies with cultured cells indicate that HSF1 not only governs part of the cytoprotection system but also plays a role in the synchronization of peripheral cells (C. Saini & U. Schibler, unpublished results).

DDDP: differential display of DNA-binding proteins

HSF1: heat-shock factor 1

Entrainment by hormonal and neuronal signals emanating from the SCN. The SCN also employs more direct signals, such as neural and humoral outputs, to synchronize peripheral clocks.

Plasma glucocorticoid hormone levels exhibit robust daily oscillations in both laboratory rodents and humans, and these cycles are driven by SCN via the hypothalamic-Pit-adrenal axis (249). Interestingly, the glucocorticoid nuclear hormone receptor is expressed in virtually all cell types, except in SCN neurons (see Reference 250 and references therein). In keeping with this expression pattern, dexamethasone, a glucocorticoid receptor agonist, acts as a strong *Zeitgeber* in vivo. Dexamethasone synchronizes rat-1 fibroblasts and causes phase shifts in peripheral oscillators but does not affect SCN rhythms (250). Thus, glucocorticoid hormones possess clock-resetting properties and represent an important phase-entrainment signal from the SCN. Due to the redundancy of synchronization pathways, the steady-state phase can be established in the absence of glucocorticoid receptors in peripheral tissues such as the liver. Nonetheless, the involvement of glucocorticoid signaling can be demonstrated by determining the kinetics of feeding-induced phase inversion. Thus, hepatocytes devoid of a functional glucocorticoid receptor gene adapt the phase much more rapidly to daytime feeding than do wild-type hepatocytes (251).

The autonomic nervous system constitutes an additional direct synchronization pathway employed by the SCN. For example, by surgically disrupting liver innervation, Buijs and coworkers (87) demonstrated that light may affect liver gene expression not only via the hormonal pathway but also via autonomic input. Hence, the autonomic nervous system may play a role in the resetting of peripheral clocks after phase shift–inducing light

exposure (87). In addition, the same group has found that plasma glucose and insulin concentrations are affected by administering glutamatergic agonists or GABAergic antagonists. The hyperglycemic effect of the GABA antagonist is absent in SCN-ablated animals. The control of daily glucose metabolism in the liver indicates an important role for GABAergic/glutamatergic SCN inputs to the hypothalamic preautonomic neurons that are connected to the liver (252). Furthermore, an elegant study by Okamura and colleagues (86) revealed that adrenal innervation is required for light-induced corticosterone secretion by the adrenal gland, thus representing a direct output from the SCN to the adrenal cortex.

Under certain circumstances, different timing cues can be in conflict with each other. We mention above that food availability during the inactivity phase will override more direct SCN-driven phase-resetting signals in the liver (194, 251). By contrast, in the submaxillary salivary gland, temporal food restriction fails to entrain circadian gene expression. However, upon sympathetic denervation, the submaxillary gland oscillators rapidly adjust their phase to the inverted feeding regimen. This finding further suggests that sympathetic inputs are important for the phase entrainment of peripheral oscillators and that multiple synchronization pathways can contribute to peripheral oscillator synchronization in the same tissue (232).

A Few Selected Outputs of Peripheral Oscillators

Hepatic clocks in glucose and lipid metabolism. Rhythmically expressed liver genes are implicated in the metabolism of fatty acids, cholesterol, bile acids, amino acids, and xenobiotics (185, 196, 199, 239). The disruption of circadian oscillator function causes alterations in metabolism (reviewed in Reference 253). For instance, mutations in the essential clock genes *Bmal1* and *Clock* lead to various metabolic disorders (254). Elegant work by Weitz and colleagues (186) suggests that functional liver clocks contribute to glucose homeostasis by driving a daily rhythm of hepatic glucose export. Using the Cre-loxP recombination strategy in mice, these authors inactivated *Bmal1* either in all cells ($Bmal1^{-/-}$) or specifically in hepatocytes (L-$Bmal1^{-/-}$). Their experiments suggest that BMAL1 function is important for the regulation of total body fat, glucose clearance, and insulin production. Animals with a liver-specific *Bmal1* disruption suffer from hypoglycemia during the inactivity phase. Yet, mice deficient in BMAL1 in all cells do not show overt problems with their resting blood sugar levels. Hence, in L-$Bmal1^{-/-}$ mice, the lack of liver rhythms—rather than just the lack of the BMAL1 transcription factor—must be the cause of impaired glucose homeostasis. Of note, Weitz and colleagues (186) have described a similar pattern of metabolic defects in $Per1^{-/-}$, $Per2^{-/-}$ double-mutant mice, which also have a disrupted clock. Moreover, a genetic linkage analysis in humans has recently shown that *hPER2* activity may be associated with blood glucose levels as well (255).

Recent studies by Le Martelot and colleagues (187) further underscore the tight link between the circadian clock and metabolism in the liver. These authors have demonstrated an important role for *REV-ERBα* in the circadian regulation of cholesterol and bile acid synthesis (187). Genetic loss- and gain-of-function experiments suggest that *REV-ERBα* participates in the circadian modulation of sterol regulatory element–binding protein (SREBP) activity and thereby in the daily expression of SREBP target genes involved in cholesterol and lipid metabolism (187). This regulation is accomplished via the cyclic transcription of *Insig2*, which encodes a transmembrane protein that sequesters SREBPs to the endoplasmic reticulum (ER) membranes and thereby interferes with the proteolytic activation of SREBPs in Golgi membranes. Probably through this mechanism, *REV-ERBα* also participates in the regulation of cyclic cholesterol-7α-hydroxylase (*Cyp7a1*) expression. Thus, the authors of this report speculate that the SREBP-dependent production of cholesterol is accompanied by the generation of oxysterols, which in turn activate

the nuclear receptor LXR and hence the LXR target gene *Cyp7a1*.

Pancreas clocks, melatonin signaling, and diabetes. An additional connection between circadian oscillators and glucose homeostasis has emerged from recent studies on melatonin receptors in the pancreas (256). Melatonin is a circulating neurohormone that is secreted predominantly by the pineal gland at night. The hormone regulates circadian rhythms by assisting the translation of photoperiodic information in the brain. Melatonin signaling is mainly mediated by two receptors, MT1 and MT2, encoded by the *MTNR1A* and *MTNR1B* genes, respectively. The strongest MT2 expression levels are detected in the retina and in the SCN. MT2-mediated melatonin signaling may indirectly regulate glucose level and insulin secretion through the SCN (257). In healthy individuals insulin secretion follows a circadian rhythm. This rhythm, generated within the islets, may be induced by melatonin, which provokes a phase shift in insulin secretion (256). Indeed, *MTNR1A* is highly expressed in pancreatic islets and is, in general, more abundant than *MTNR1B*. The presence of *MTNR1B* was recently confirmed in both pancreatic islets and sorted β-cells, suggesting a possible direct role of MT2 in the regulation of insulin secretion (258). Genetic studies in humans have revealed a possible link between *MTNR1B* allele variants and hyperglycemia, impaired early-phase insulin secretion, and β-cell function (258, 259). Thus, *MTNR1B*, expressed in human pancreatic β-cells, may represent an important connection between circadian clocks and glucose homeostasis, and its detailed analysis may open new avenues for the treatment of type 2 diabetes. Recent studies on transgenic mice underscore the involvement of melatonin signaling in islet function. Analysis of circadian gene expression in pancreas (and liver) of melatonin receptor knockout mice revealed changes in both phase and amplitude. Moreover, an upregulation of insulin secretion was detected in isolated islets of MT1 and MT2 single-knockout mice and MT1/MT2 double-knockout animals, confirming the negative action of melatonin signaling on insulin secretion (260).

CONCLUSIONS AND PERSPECTIVES

Remarkable progress has been made in our understanding of the mammalian circadian timing system during the past few years. The molecular oscillator model has been considerably revised and amended. In particular, a plethora of post-translational regulatory mechanisms have been shown to contribute critically to proper clock function in addition to the canonical TTFL. Furthermore, the evidence for a tight coupling of circadian gene expression to metabolic cycles in mammalian cells, originally proposed by Rutter and colleagues (241), is now stronger. A new level of complexity has recently been added to the mammalian circadian timing system by findings that microRNAs participate in the control of posttranscriptional clock output pathways (205, 261). However, we must consider that core clock proteins also perform noncircadian functions. That is, when we study organisms with mutations in core clock genes, we must discriminate between clock gene phenotypes and clock phenotypes. For example, the deficiencies of clock mutant mice in bone formation, tumor surveillance, and metabolic pathways may have no causal relationship with the circadian clock. Indeed, the rigorous discrimination between clock function and clock gene function is not a trivial matter and to date has been accomplished in only a few isolated cases by so-called resonance studies (262–264). Now that period-length mutations are available for laboratory mice (265 and references therein), such studies could be performed for mammalian organisms. Even in the absence of such evidence, the facts remain that hundreds of genes are expressed in a cyclic manner and that most physiology follows a daily rhythm. Alas, this knowledge is largely ignored in the clinic, and only a few pioneers are making efforts to explore this temporal information in designing better therapies (266).

The synchronization pathways of peripheral clocks by the SCN remain largely unknown. Many signaling pathways are expected to participate in the phase entrainment of peripheral clocks. Hence, the elimination of a single pathway is unlikely to significantly affect the steady-state phase of circadian clocks. A more sensitive approach would be the recording of phase-shifting kinetics. Ideally, such recording would be performed at a high temporal resolution in living animals. It will thus be of utmost importance to develop novel noninvasive whole-body imaging techniques allowing the recording of central and peripheral gene expression patterns in freely moving animals.

SUMMARY POINTS

1. The main circadian oscillator in the brain is located in the suprachiasmatic nuclei (SCN). Secondary oscillators exist in the brain.
2. Light adjusts the phase of the SCN oscillator to the environmental light-dark cycle.
3. Neural and humoral signals from the SCN can synchronize central and peripheral oscillators and vice versa.
4. In the periphery, circadian clocks are functional in most body cells.
5. Peripheral oscillators are self-sustained; cell-autonomous; and resilient to cell division, to acute stress, and to fluctuations in temperature and general transcription rate.
6. The combination of environmental inputs—such as daily fasting-feeding cycles, temperature cycles, the cellular redox state, and direct neural and humoral signals from the SCN—represents essential entrainment cues for the peripheral clocks.

DISCLOSURE STATEMENT

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