Peripheral Circadian Oscillators in Mammals

Steven A. Brown and Abdelhalim Azzi

Abstract Although circadian rhythms in mammalian physiology and behavior are dependent upon a biological clock in the suprachiasmatic nuclei (SCN) of the hypothalamus, the molecular mechanism of this clock is in fact cell autonomous and conserved in nearly all cells of the body. Thus, the SCN serves in part as a "master clock," synchronizing "slave" clocks in peripheral tissues, and in part directly orchestrates circadian physiology. In this chapter, we first consider the detailed mechanism of peripheral clocks as compared to clocks in the SCN and how mechanistic differences facilitate their functions. Next, we discuss the different mechanisms by which peripheral tissues can be entrained to the SCN and to the environment. Finally, we look directly at how peripheral oscillators control circadian physiology in cells and tissues.

Keywords Feeding • Fibroblast • HPA axis • Temperature

1 Introduction: The Discovery of Peripheral Clocks

The basic unit of circadian timekeeping is the cell. Because clocks had been discovered in many unicellular organisms, it was obvious even half a century ago that individual cells *can* possess machinery to tell time. Nevertheless, in 1972, lesion studies identified a single tissue, the suprachiasmatic nuclei (SCN) of the hypothalamus, as necessary for circadian physiology and behavior in mammals (Stephan and Zucker 1972), and soon thereafter, central clock tissues or cells were also identified in birds (Takahashi and Menaker 1979), reptiles (Janik et al. 1990), and fruit flies (Liu et al. 1988). Therefore, most investigators twenty years

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ago imagined a centralized circadian timekeeping system through which signals from a master clock tissue orchestrated different diurnal processes in metazoans (Kawamura and Ibuka 1978).

The discovery of specific clock genes expressed in most cells created the possibility and the motivation to question this hypothesis. If clock function were based upon feedback loops of transcriptional repression (Hardin et al. 1990) and the genes and proteins involved in this mechanism were conserved in all metazoans and present in all tissues, then it would be possible to envision cell-autonomous clocks even in highly complex organisms. Indeed, in 1995, Welsh and colleagues showed that dispersed neurons of the suprachiasmatic nucleus each contained independently ticking clocks, as evinced by slightly different period lengths of spontaneous electrical activity. Timekeeping continued even when this electrical activity was blocked (Welsh et al. 1995). Similar clocks were also found in cultured retina (Tosini and Menaker 1996). Hence, in mammals as in bacteria, circadian timekeeping could be cell autonomous.

The finding of clock genes also permitted the invention of new technologies to probe clock function at a molecular level. By creating DNA "reporters" that use clock gene sequences to drive expression of bioluminescent or fluorescent proteins in individual cells, investigators could for the first time ask about clock gene function in different parts of an organism separately and noninvasively. Such a technology applied to fruit flies showed that different fly pieces contained autonomous clocks that functioned independently of "master clock" pacemaker neurons in the fly head (Plautz et al. 1997), and even cultured mammalian skin fibroblast cells contained autonomous clocks that tick in culture, completely independent of the SCN (Balsalobre et al. 1998).

The existence of peripheral clocks not only proved a significant boon to the understanding of clock mechanisms – now they could be studied in culture or in easily accessible tissues (Cuninkova and Brown 2008) – but also provoked a paradigm shift: maybe master clock tissues served not to send separate signals for different aspects of physiology but rather to synchronize peripheral clocks in other tissues, which in turn autonomously controlled circadian physiology.

The last decade has shown that both purely centralized and purely peripheral models are too simple. In reality, some aspects of mammalian physiology are controlled by peripheral clocks and others directly by central signals. Similarly, peripheral clocks sometimes accept signals from the SCN and sometimes take cues directly from their environment, and their entrainment has proven to be a web of both direct and indirect signals that can even vary from tissue to tissue. In this chapter, we shall begin by considering the molecular mechanisms of peripheral clocks and their similarities and differences to central clocks. Subsequently, we consider the mechanisms by which they are entrained and finally the complex physiology that they control in mammals.

2 Peripheral Clock Mechanisms

As a whole, the mechanism of circadian clocks in peripheral cells is remarkably similar to that of the "master" clock in SCN cells. For example, in humans, circadian period length measured in peripheral skin fibroblasts in vitro is directly

proportional to the circadian period of SCN-controlled behavior in the same subjects (Pagani et al. 2010). Moreover, analysis of peripheral and central clocks in mice deficient for individual clock proteins showed clearly that the broad outline of clock mechanism is the same in fibroblasts as in SCN (Yagita et al. 2001): feedback loops of transcription, translation, and posttranslational modification control most studied aspects of cellular circadian physiology.

As described in previous chapters, these loops are thought to be based upon a set of transcriptional activators (the CLOCK and BMAL1 proteins), which activate a set of repressor genes (the period loci *Per1-3* and cryptochrome loci *Cry1-2*), whose protein products repress their own transcription. In a separate loop, the nuclear receptor ROR and REV-ERB proteins activate and repress the *Bmal1* gene, respectively. Connecting these loops, the *Rev-Erbα* gene is itself regulated by CLOCK and BMAL1. (See Buhr and Takahashi 2013) for a detailed and referenced description of these molecular mechanisms.) In spite of this close overall similarity, on the level of gene expression, the cell-autonomous clocks ticking in each tissue have a slightly different set of core and associated clock loci directly involved in their timekeeping mechanism, and these differences have significant ramifications for the physiology that they direct.

2.1 Complements of Clock Genes and Proteins Vary from Tissue to Tissue

Although most identified "core clock genes" are present in most tissues, in some cases homologous genes assume different tissue-specific functions. For example, a deletion of one of the three mammalian *period* homologs, *Per3*, has only the subtlest of effects on the central clock mechanism (Shearman et al. 2000). However, some specific peripheral tissues like pituitary, liver, and aorta show a pronounced effect of *Per3* deletion on clock period length in tissue explants and clock phase in vivo (Pendergast et al. 2011). Therefore, it is likely that PER3 plays an important role in clock mechanism in some tissues but is redundant in others.

A similar functional overlap exists between CLOCK and its homolog NPAS2. In the SCN, loss of CLOCK protein is probably compensated by the presence of NPAS2, so that mice deficient for the *Clock* gene are behaviorally rhythmic (Debruyne et al. 2006), but in most peripheral tissues, CLOCK deletion leads to arhythmicity of circadian oscillators in tissue explants (DeBruyne et al. 2007a, 2007b) as well as in vivo (Dallmann et al. unpublished). In reverse, NPAS2 is believed to be important for the clock in the forebrain (Reick et al. 2001).

In addition, various auxiliary factors can play important tissue-specific roles in clock function. For example, oligophrenin 1 appears to regulate circadian oscillations in the hippocampus by interacting with REV-ERBα and modifying its transcriptional repression activity (Valnegri et al. 2011). Similarly, the two isoforms of AMP kinase (which are thought to phosphorylate CRY proteins

(Lamia et al. 2009)) have dramatic but tissue-specific effects upon circadian oscillator function (Um et al. 2011). Finally, a range of nuclear receptor proteins can interact with clock proteins such as REV-ERBα (itself a nuclear receptor) and PERs (Schmutz et al. 2010), and the tissue-specific distribution of such receptors likely leads to tissue-specific differences in circadian function (Teboul et al. 2009).

More broadly, both in vivo and in vitro, different mouse tissues show different circadian phases in tissue explants (Yamazaki et al. 2000; Yoo et al. 2004). While a portion of this variation is undoubtedly due to differences in entrainment signals, another portion is probably due to intrinsic variation in period from tissue to tissue – with shorter periods leading to earlier phases. For example, a five-hour phase difference is observable between liver and spleen, and nearly eight hours between liver and gonadal adipose tissue. Supporting a tissue-intrinsic mechanism for these phase differences, free-running period in tissue explants differed by 2–4 hours between liver and the other two tissues (Pendergast et al. 2012), again pointing to subtle tissue-specific differences in free-running clock mechanism. Intriguingly, testis is the only mammalian tissue that, so far, has not been shown to harbor a self-sustained clock (Alvarez et al. 2003).

These results demonstrate that each tissue can have its own complement of core clock genes that may vary in abundance or function. This sometimes subtle variation could lead to pronounced tissue specificity in clock-controlled output genes, as discussed later.

2.2 *Peripheral Tissues Lack Neuropeptidergic Signaling that Promotes Network Synchrony*

The second major difference between central and peripheral clocks relates to their network properties. Cultured fibroblasts and tissue explants from peripheral organs like liver, spleen, kidney, heart, and lung exhibit robust circadian oscillations in gene expression, at least initially (Yamazaki et al. 2000). However, all of these peripheral clocks have in common that their oscillations damp rapidly in culture. In contrast to peripheral tissues, SCN explants are capable of generating rhythmic gene expression and electrical activity for weeks or even years in culture. Interestingly, this damping has little to do with the cell-autonomous properties of peripheral and SCN cells. For example, cultured fibroblasts show persistent oscillations in culture that exceed the robustness of individual SCN neurons (Welsh et al. 2004). In fact, even though intact SCN explants show remarkably persistent oscillations, dispersed SCN neurons show very intermittent oscillations (Webb et al. 2009). The difference between SCN and periphery lies in coupling: whereas peripheral cells oscillate mostly independently of one another in vitro (Nagoshi et al. 2004; Welsh et al. 2004), SCN neurons possess specific mechanisms to maintain synchrony as a population and even appear to require them for stable oscillations.

Three different mechanisms appear to be used for coupling: synaptic potentials, electrical synapses, and neuropeptidergic signaling. The first two are common to most neurons: inhibition of voltage-dependent sodium channels (Welsh et al. 1995), GABAergic signaling (Albus et al. 2005), or gap junctions formed by connexins (Long et al. 2005; Shinohara et al. 2000) reduces the synchrony of SCN neuron populations in vitro. The third mechanism is more unique: neuropeptidergic coupling. Circadian secretion of vasoactive intestinal peptide (VIP) by a subset of SCN neurons is perceived as a paracrine timing cue by neighboring cells expressing its receptor, VPAC2. Loss of this coupling mechanism, either by ablation of VIP or of VPAC2, abolishes the circadian firing rhythm of a subset of SCN neurons, and mice harboring this mutation are therefore incapable of normal circadian rest/activity rhythms (Aton et al. 2005; Colwell et al. 2003). In total, it is likely that three neurotransmitter systems play overlapping roles in this coupling: primarily VIP, with contributions from arginine vasopressin (AVP) and gastrin releasing peptide (GRP) (Maywood et al. 2011). Other neurotransmitter systems may also play a role through tonic signaling. For example, the PAC1 receptor is normally involved in the response of the SCN to light, but deletion of the PAC1 receptor also changes circadian expression of VIP (Georg et al. 2007).

Although circadian peptidergic signaling is so far believed to be unique to the SCN, other mechanisms are certainly present in other tissues—e.g., sodium channels in heart or gap junctions in liver—and may be useful to achieve some degree of coupling. For example, in SCN-lesioned animals, individual organs still maintain some degree of circadian synchrony in clock gene expression, although this varies both among animals and among organs (Yoo et al. 2004). Nevertheless, it is universally accepted that this coupling is much less than in SCN. At a cellular level, there are two consequences of this lack. First, clock mechanisms in peripheral cells are more susceptible to mutation. For example, disruptions of individual nonessential clock genes have larger effects upon clock function in cultured fibroblasts than upon behavior in the same mice (Brown et al. 2005; Liu et al. 2007). This observation is clearly a consequence of greater coupling in SCN cells because larger effects can also be seen in dissociated SCN cells vs. intact slices (Liu et al. 2007). Secondly, the lesser coupling of peripheral cells permits greater phase shifting, making peripheral oscillators less "rigid." At least in vitro, this means that clocks from peripheral tissues (e.g., lung) can entrain to more extreme zeitgeber cycles, whereas the more rigid SCN clock will instead "free run" at its own intrinsic period (Abraham et al. 2010).

3 Entrainment of Peripheral Clocks

As mentioned in the previous paragraph, one consequence of mechanistic differences between oscillators in peripheral tissues and those in the SCN is variation in susceptibility to entrainment signals. Indeed, the most fundamental difference between central and peripheral oscillators lies in the signals to which they respond. A key characteristic of peripheral oscillators is their ability to respond

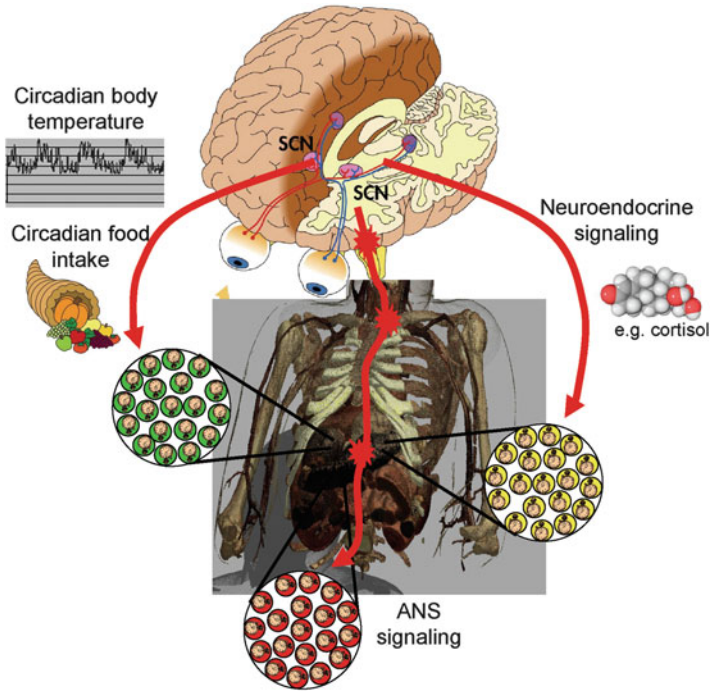

Fig. 1 Signals from SCN to peripheral oscillators. Synchronizing signals include direct nervous signals from the autonomous nervous system, neuroendocrine signals like glucocorticoids, and indirect signals such as circadian body temperature and food intake, which are both determined under normal circumstances by patterns of activity and rest (This diagram was adapted from original drawings by N. Roggli, as well as images from the Visible Human Project of the USNLM)

to SCN-driven timing signals and that of the "master clock" in the SCN is its blindness to these signals and instead its entrainment to a limited range of environmental stimuli. In general, whereas the SCN responds primarily to environmental light – a phenomenon described by Slat et al. (2013) and Roenneberg et al. (2013) – peripheral clocks are thought to respond to a complex and redundant combination of direct nervous stimuli, hormonal signals, and indirect activity-directed signals such as body temperature and the timing of food intake. These signals are described below and summarized in Fig. 1.

3.1 Entrainment by Direct Nervous Stimuli

SCN neurons project throughout the brain and, via their spontaneous circadian firing activity, are thought to provide signals for a wide variety of circadian behaviors. For example, projections to the subparaventricular zone (SPVZ) are responsible for circadian rhythms of locomotor activity via multiple hypothalamic arousal systems

(Abrahamson and Moore 2006). Similarly, reduced firing activity of the SCN during the late sleep phase directly affects osmoregulatory neurons that control vasopressin release and thereby suppress urination (Trudel and Bourque 2012). GABAergic input from the SCN to the paraventricular nucleus (PVN) controls circadian glucose production in the liver (Kalsbeek et al. 2004) and melatonin production in the pineal gland (Kalsbeek et al. 2000). An anatomically separate stimulatory output from the SCN is also necessary for correct circadian melatonin production (Perreau-Lenz et al. 2003). For the regulation of sleep and arousal, SCN projection to the locus coeruleus (LC) via the dorsomedial hypothalamus (DMH) is believed to play a central role, and LC neuronal activity displays a circadian firing rhythm (Aston-Jones et al. 2001). For the moment, although projections from the SCN to other brain regions directly regulate neural activity in target areas, it is unclear whether they also regulate cell-autonomous circadian clocks in target cells.

Beyond the brain, the autonomous nervous system plays a direct role in communicating circadian SCN timing signals to multiple tissues. For example, from the PVN, SCN signals travel via the autonomous nervous system to the liver to control glucose production (Kalsbeek et al. 2004). A multisynaptic autonomic nervous connection also exists between SCN and heart to regulate cardiac rate in circadian fashion (Scheer et al. 2001) and to the adrenal gland to regulate both circadian and light-dependent corticosterone production (Ishida et al. 2005). These examples are likely to represent only a small portion of physiology directly mediated by autonomous SCN connections: in total, sympathetic efferents have been documented for brown adipose tissue, thyroid gland, kidney, bladder, spleen, adrenal medulla, and adrenal cortex. Parasympathetic nervous system innervation of the thyroid, liver, pancreas, and submandibular gland has also been reported. Thus, some tissues are even innervated both sympathetically and parasympathetically by the SCN. Again, the functional implications of many of these connections are as yet uncertain (Bartness et al. 2001).

From the literature cited above, it is clear that at least some direct nervous efferents, both sympathetic and parasympathetic, can control circadian physiology. Based upon the analysis of clock gene expression in peripheral organs of hamsters whose two suprachiasmatic nuclei showed different phases, it is also clear that such signals can also play a role in the phase entrainment of peripheral clocks in some peripheral organs, like skeletal muscle, adrenal medulla, and lung, but not in others like liver or kidney (Mahoney et al. 2010). Given the ability of several neurotransmitter classes to act via pathways that phase-shift cellular clocks (e.g., cAMP and MAP kinase cascades), such control would not be surprising. Moreover, most of the direct nervous connections studied—either through hormones or effects upon behavior—can also influence peripheral clocks indirectly, as discussed next.

3.2 Entrainment by Peptides and Hormones

A second major path controlling circadian clocks is hormonal. Although nervous efferents from the SCN clearly play an important role, it has long been clear that this role is not essential, at least for the control of diurnal behavior. Lesion of the

SCN results in arrhythmic behavior, but implantation of fetal SCN tissue can rescue circadian locomotor activity, even when such an implant is encased in porous plastic (Silver et al. 1996). Therefore, diffusible factors from the SCN are capable of entraining circadian behavior. So far, two diffusible timing factors have been identified: transforming growth factor alpha (TGFα) (Kramer et al. 2001) and prokineticin 2 (PK2) (Cheng et al. 2002). These signaling proteins alter locomotor activity when injected chronically into the third ventricle, and both are secreted in circadian fashion by the SCN. While neither factor directly resets peripheral clocks, their control of activity provides indirect signals that do, as discussed below. Multiple other factors might also be important: recent advances in analytical technologies have enabled direct, high-resolution peptidomic profiles of rat SCN neurons, which produce a total of 102 endogenous peptides (Lee et al. 2010).

Another way by which the SCN entrains circadian physiology and gene expression in peripheral clocks is via the pituitary–adrenocortical axis, specifically via glucocorticoids, a class of steroid hormones that bind to the glucocorticoid receptor (GR). These hormones are secreted in daily fashion, and their receptors (GR) are expressed in most peripheral cell types, but not in SCN neurons. In addition to the critical role that glucocorticoids play in metabolism, it has been shown that in vitro and in vivo application of the glucocorticoid analog dexamethasone induces *Per1* expression in RAT1 fibroblasts and shifts or resets the phase of circadian gene expression in peripheral tissues but not SCN. Glucocorticoids are redundant with other timing signals because mice lacking GR in the liver still express genes in circadian manner in this organ (Balsalobre et al. 2000a).

Beyond glucocorticoids, at least in vitro, input to three other classes of signaling pathways has been identified as capable of independently phase-shifting peripheral circadian clocks: cAMP and MAP kinases, protein kinase C, and calcium signaling (Balsalobre et al. 2000b). Multiple signaling agents acting through these pathways have been shown to induce and synchronize circadian clocks in vitro, including endothelin-1 (Yagita et al. 2001), fibroblast growth factor, epidermal growth factor (Akashi and Nishida 2000), forskolin (Yagita and Okamura 2000), glucose (Hirota et al. 2002), and prostaglandin E2 (Tsuchiya et al. 2005). Based upon different phase shifting profiles, these various agents appear to intersect the known circadian clockwork in at least two different nodes, one showing rapid induction of the clock gene *Per1* and the other slow and weak induction of it (Izumo et al. 2006).

How this myriad of signals controls circadian phase in peripheral oscillators in vivo is until now unclear: only prostaglandin E2 and dexamethasone have been shown to shift circadian clocks acutely in peripheral organs when injected into mice (Balsalobre et al. 2000b; Tsuchiya et al. 2005), and all implicated pathways are essential for proper development, rendering conventional loss-of-function studies difficult. Nevertheless, in the case of glucocorticoid signaling, conditional and tissue-specific disruptions have allowed investigators to show unambiguously that glucocorticoid signaling plays an important role in the timing of circadian physiology, gene expression, and clock phase, especially in the liver (Kornmann et al. 2007; Reddy et al. 2007). Similar approaches with other signaling pathways should yield important information about roles of other hormone-dependent signaling cascades in peripheral circadian physiology.

3.3 Entrainment by Indirect Cues: Temperature and Feeding

In addition to direct cascades leading from the SCN to entrain peripheral clocks, there exist two important indirect cues that arise as a consequence of circadian behavior: temperature and food intake. Even in homeotherms such as mammals, circadian rhythms of activity and metabolism direct subtle fluctuations in body temperature (1–4 degrees Celsius, depending upon the organism). Both in cells and in living mammals, these rhythms are sufficient to entrain peripheral circadian oscillators (Abraham et al. 2010; Brown et al. 2002; Buhr et al. 2010), possibly via circadian oscillations in the activation of the same transcription factors that regulate the response of cells to acute heat shock (Reinke et al. 2008).

Similarly, patterns of feeding can directly entrain clocks in peripheral organs: inversion of the timing of food availability will inverse the timing of peripheral clocks, independently of the suprachiasmatic nucleus (Damiola et al. 2000; Stokkan et al. 2001). The speed as well as the degree of phase shift induced by inversed feeding varies among different organs. For example, mRNA of the clock gene *Dbp* examined in mice fed only during the light phase shows a strong temporal difference in liver, kidney, heart, and pancreas, whereas in mice fed during the dark phase, the accumulation of Dbp mRNA was around ZT14 to ZT18 in all analyzed tissues.

The mechanism by which peripheral oscillators can be entrained by food remains unclear. Since glucose itself can reset circadian clocks in cultured cells, it has been suggested that this simple food metabolite could play a role (Hirota et al. 2002). More broadly, circadian clock function is regulated in a variety of ways by cellular redox potential, which itself fluctuates via metabolism. The dimerization of CLOCK and BMAL1 and their binding to *cis-acting* DNA elements is itself regulated by redox potential, at least in vitro (Rutter et al. 2001), and the NAD +−dependent histone deacetylase SIRT1 directly interacts with the CLOCK: BMAL1 heterodimer to facilitate deacetylation and degradation of PER2 (Asher et al. 2008) and deacetylation of BMAL1 and local histones (Nakahata et al. 2008). At the same time, the NAD+−dependent ADP-ribosylate PARP1 interacts with CLOCK to ADP-ribosylate it and interfere with its binding, a process also important for correct entrainment to feeding (Asher et al. 2010). Another method of synchronizing circadian clocks to metabolism is probably mediated by cryptochrome clock proteins, which are phosphorylated and targeted for degradation by AMP-dependent kinase (AMPK), an enzyme regulated by cellular ATP/AMP balance (Lamia et al. 2009).

Other possible contributors to food-dependent entrainment of peripheral clocks are feeding-dependent hormones. Although glucocorticoids are obviously important to metabolic regulation, they appear to play no role. In fact, their signal opposes that of inversed feeding, and mice with tissue-specific loss of glucocorticoid receptor entrain much faster to changes in feeding schedules (Le Minh et al. 2001). By contrast, the hormone ghrelin might contribute to clock entrainment by feeding. Ghrelin is a 28-amino acid peptide produced mainly by P/D1 cells

covering the stomach and epsilon cells of the pancreas. It has also been reported that ghrelin levels exhibit a circadian rhythm and follow feeding schedules. Thus, it has been postulated that ghrelin-secreting cells are themselves entrained by feeding and then their hormonal signal serves as a messenger to other cells, both in the brain and in other peripheral tissues (LeSauter et al. 2009). Importantly, ghrelin can also modify SCN phase or its response to light both in vivo and in vitro, making it a candidate for broader modifications in circadian behavior in response to restricted feeding (Yannielli et al. 2007; Yi et al. 2008).

3.4 How the SCN Avoids Entraining Itself

The SCN sends a wide diversity of signals to entrain peripheral circadian physiology. However, at least theoretically, it is important that it remains insensitive to such signals. Otherwise, strong damping of oscillations would be predicted. Several biological mechanisms have been elucidated to achieve this end and render the SCN blind to the entrainment signals that it sends to peripheral tissues. For nervous signals, the problem is easily resolved: by definition, such signals are directional. For hormonal stimulation, the problem is more difficult because many hormones can cross the blood–brain barrier. Interestingly, however, the best-characterized hormone for entrainment of peripheral clocks, glucocorticoid hormone, has few or no receptors on SCN cells (Balsalobre et al. 2000a).

For indirect signals like temperature and food, the problem is even more complicated: heat shock factor, for example, is universally present in cells, as are sirtuins. In the case of temperature variation, the SCN is clearly not entrained like peripheral cells: inversing circadian body temperature fluctuations in mice by environmental temperature cycles will inverse circadian gene expression in peripheral cells (including non-SCN brain regions, in spite of innervation from the SCN). The SCN itself, however, is unaffected (Brown et al. 2002). Exactly why the SCN is resistant to such entrainment signals is an important question that recent studies have helped to clarify. Interestingly, the "temperature resistance" of the SCN is a network property and not a cell-autonomous one—i.e., SCN neurons in an intact network are insensitive to temperature signals, but dissociated SCN neurons are not (Buhr et al. 2010). The most likely explanation for this phenomenon is that SCN network properties render its clock more "rigid," which would permit entrainment to environmental signals within only a narrow range. As a practical result, sudden dramatic changes in period or phase of temperature signals would be ignored (Abraham et al. 2010). The latter model could also explain failure to entrain to sudden changes in feeding signals as well: for mice subjected to inverted feeding cycles, the SCN remains unshifted even as peripheral clocks change up to 180 degrees in phase (Damiola et al. 2000; Stokkan et al. 2001). In the case of the latter model, however, a specific exception would have to be made for light-dependent phase shifting: for mice subjected to sudden "jet lag" with shifts in light and via activity rhythms food and temperature cycles also, different organs shift at different

rates, but the SCN is among the most rapid to adopt the new phase (Davidson et al. 2008; Yamazaki et al. 2000).

4 Physiological Control by Peripheral Circadian Clocks

A large number of physiological processes are under circadian control. These include xenobiotic detoxification, lipid metabolism, renal plasma flow and urine production, cardiovascular parameters such as blood pressure and heart beat rates, and even many aspects of immune function (Gachon et al. 2004). The cell-autonomous nature of the circadian clock, coupled with its hierarchical entrainment structure in mammals, would suggest that circadian physiology in peripheral tissues is largely controlled by peripheral oscillators. In fact, this statement is only partially true. Certainly, many aspects of diurnal physiology in peripheral tissues are directly dependent upon circadian clocks in these tissues. Other aspects, however, are controlled by circadian autonomous nervous or hormonal signals indirectly originating from the SCN.

4.1 Cell-Autonomous Circadian Physiology

As described above and in previous chapters, the canonical circadian clock mechanism is controlled by transcriptional feedback loops in which clock proteins bind to *cis*-acting DNA elements to activate or repress the expression of other clock proteins. Interestingly, however, these same elements are present throughout the genome and regulate clock-controlled genes as well (Ripperger et al. 2000). Therefore, they probably serve as one of the principal conduits by which peripheral circadian physiology is directed. Such rhythmic transcriptional control is believed to be generated through three principal binding motifs in promoter regions: E-boxes, D-boxes, and Rev-Erbα/ROR-A response elements (RREs) (Ueda et al. 2005; Minami et al. 2013). Various combinations of these elements are capable of generating a wide variety of phase profiles. In total, about ten percent of transcripts in all peripheral tissues are regulated in circadian fashion (Panda et al. 2002; Storch et al. 2002; Reddy 2013).

Recently, genome-wide technologies—ChIPseq to identify binding sites for particular proteins on a genomic scale, RNAseq to identify sequences present in all transcripts, etc.—have dramatically increased our knowledge of how clock factors control gene expression in peripheral tissues and of which pathways are controlled (Reddy 2013). For example, genome-wide analyses of binding targets of BMAL1 (Hatanaka et al. 2010; Rey et al. 2011) and multiple other circadian clock factors in liver (Koike et al. 2012) have clarified not only which pathways are controlled (particularly carbohydrate and lipid metabolism) but also how different regulatory elements contribute to this regulation. Similar profiling of REV-ERBα

and REV-ERBβ targets has shown liver regulation of both core circadian clock and metabolic networks by both proteins (Cho et al. 2012).

In the liver, whose circadian physiology has been particularly well studied, one example of peripheral clock-directed transcriptional control is furnished by xenobiotic metabolism pathways. Here, circadian transcription of PAR-B-ZIP (proline- and acidic amino acid-rich basic leucine zipper) transcription factors like *Dbp* (D-element binding protein) is controlled by the clock proteins CLOCK and BMAL1 via *cis*-acting E-box elements (Ripperger et al. 2000). PAR-B-ZIP factors bind to D elements in the promoter of the constitutive androstane receptor (CAR) gene, which in turn controls circadian expression of many cytochrome P450 isoforms that directly regulate metabolism of a wide variety of xenobiotics (Gachon and Firsov 2010; Gachon et al. 2006). This cascade of circadian transcription factors is diagrammed in Fig. 2. The same three PAR-B-ZIP factors also play a key role in directing circadian lipid metabolism by controlling expression of the *PPARα* (peroxisome proliferator-activated receptor alpha) gene (Gachon et al. 2011). Liver glucose metabolism is also strongly regulated by the cell-intrinsic liver circadian clock. In fact, peripheral clock-regulated hepatic glucose export probably counterbalances feeding-driven rhythms of daily glucose ingestion in order to maintain relative homeostasis (Lamia et al. 2008). Although the control mechanisms described above highlight transcriptional mechanisms based upon repression and initiation of transcriptional initiation, an increasing number of studies suggest that other later steps in transcription (Koike et al. 2012), including RNA export or stability (Morf et al. 2012), transcriptional termination (Padmanabhan et al. 2012), and splicing (McGlincy et al. 2012), also play important roles. Since the percentage of circadian proteins in liver is greater than the number of circadian transcripts (Reddy et al. 2006), it is likely that entirely posttranscriptional circadian regulatory mechanisms are also operative.

Although these studies were done mostly in liver, peripheral circadian clocks also play a strong role in many other organs. For example, the strong circadian rhythmicity of renal function has long been known (Minors and Waterhouse 1982). However, core clock transcripts like *Clock, Bmal1, Npas2, Per1-3,* and *Cry1-2* are expressed in the distal nephron with robust oscillations, and mice lacking either CLOCK or PAR-B-ZIP factors show significant changes in renal expression of key regulators of water and sodium balance, as well as changes in sodium excretion itself (Zuber et al. 2009). Therefore, kidney-intrinsic circadian oscillators are likely to play a key role in physiological regulation by this tissue. Likewise, circadian clocks in macrophages (Keller et al. 2009) and T cells (Fortier et al. 2011) govern inflammatory immune responses, and the clock protein REV-ERBα appears to play a specific role in selectively regulating inflammatory cytokines (Gibbs et al. 2012).

In other tissues, the retinal circadian clock is essential to circadian oscillations of light response in the inner retina (Storch et al. 2007). Moreover, arterial transplants from animals lacking circadian clocks develop atherosclerosis in transplanted blood vessels, proving a role for autonomous circadian clocks here as well (Cheng et al. 2011). Circadian clock ablation in pancreatic islets results in diabetes due to defects in coupling of beta cell stimulus to insulin secretion

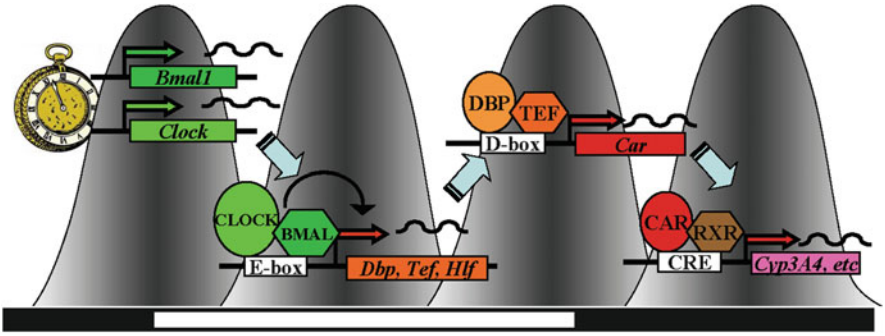

Fig. 2 Circadian transcription factor cascades determining xenobiotic metabolism in the liver. The transcriptional activators CLOCK and BMAL1, parts of the fundamental mechanism of the circadian oscillator, activate transcription of genes encoding the PAR-B-ZIP transcription factors DBP, TEF, and HLF. These proteins in turn activate transcription of the constitutive androstane receptor (CAR). (For clarity, only DBP and TEF are pictured.) The CAR protein then activates transcription of cytochrome P450 loci, either alone or as a dimer with RXR, the retinoid X receptor

(Marcheva et al. 2010). In cardiac tissues, peripheral clocks control expression of multiple kinases and ion channels, and cardiac clock mutation changes physical activity (Ko et al. 2011) and cardiac triglyceride metabolism (Tsai et al. 2010). Mutation of clocks in circulatory epithelium eliminates circadian rhythms in thrombogenesis (Westgate et al. 2008). Circadian transcriptome analyses of skeletal muscle and adipocyte tissues in tissue-specific clock-deleted animals show the regulation of at least 400 genes by muscle cell clocks and 660 by adipocyte clocks (Bray and Young 2009), suggesting that considerable circadian physiology in these tissues is peripherally regulated. Similarly, direct clock control of NAD+ salvage also implies that regulation of cellular metabolism is peripherally controlled (Nakahata et al. 2009). Finally, circadian clocks in adrenal tissue are essential for circadian production of glucocorticoids (Son et al. 2008), and clocks in target tissues possibly even control circadian glucocorticoid receptor expression (Charmandari et al. 2011). Similarly, circadian clock control of adrenal aldosterone production via the enzyme Hsd3b6 is an important regulator of blood pressure (Doi et al. 2010). For both of these hormones, their circadian biosynthesis is under control of adrenal circadian clocks, even if stimulation of the adrenal gland is sympathetically driven.

4.2 Direct Endocrine Control of Circadian Physiology

Although a considerable amount of circadian physiology is directed by peripheral clocks, another portion is not. A large number of circadian endocrine factors are able to directly elicit circadian physiological responses without contributions from peripheral clocks in target tissues. For example, tissue-specific disruption of

circadian clock function in liver and in other tissues has revealed that a portion of circadian gene expression is also systemically driven by neuroendocrine signals, most notably glucocorticoids. Disruption of liver clocks by interfering with *Bmal1* expression in vivo revealed 31 genes whose expression was still circadian (Kornmann et al. 2007). Comparable results have been seen in other tissues like muscle, heart, and fat (Bray and Young 2009). Similarly, glucocorticoid signaling is not only able to synchronize peripheral circadian oscillators (Balsalobre et al. 2000a), but it can also independently control 60% of the circadian transcriptome (Reddy et al. 2007). Interestingly, this control appears to be modulated by a direct interaction between glucocorticoid receptors and the cryptochrome clock proteins (Lamia et al. 2011). Other nuclear-receptor-coordinated physiology may also be modulated by direct interactions with clock proteins: PER2 has been shown to interact with PPARα and REV-ERBα (Schmutz et al. 2010). In the brain, direct interactions between REV-ERBα and oligophrenin 1 appear to play an important role in hippocampal circadian clocks and affect localization of REV-ERBα (supposedly a nuclear transcription factor) to synapses (Valnegri et al. 2011).

Circadian activity of the hypothalamic–pituitary–adrenal (HPA) axis is only one aspect of endocrine control of peripheral circadian physiology. A second example of endocrine regulation is the hormone melatonin, which exerts diverse circadian effects upon sleep and inflammation (Hardeland et al. 2011). Because so many endocrine factors are secreted in circadian fashion, numerous other examples exist, ranging from immune cytokines like TNFα to growth hormone and gonadal steroids like testosterone (Urbanski 2011). The circadian physiology that they control is considered in more detail (Kalsbeek and Fliers 2013).

4.3 Indirect Control of Circadian Physiology

Through its regulation of activity cycles and feeding, the SCN can not only send endocrine signals that regulate peripheral circadian clocks but also directly control circadian physiology. For example, in the mouse liver, only a small proportion of transcripts displayed circadian expression patterns in the absence of food, and conversely, temporally restricted feeding could restore circadian transcription of a sizable fraction of the circadian transcriptome even in the absence of functional liver clocks (Vollmers et al. 2009). Similarly, of 2,032 cortical transcripts under circadian control, only 391 remained rhythmic during sleep deprivation (Maret et al. 2007), thereby implying an essential contribution of rest–activity rhythms to circadian physiology and gene expression, at least in some tissues. Another recent study demonstrated how temperature fluctuations could drive circadian expression of some factors like cold-induced RNA-binding protein (CIRP) independently of the core circadian clock, reinforcing its function (Morf et al. 2012). Altogether, the exact contributions of these indirect cues in circadian physiology remain an exciting new aspect of clocks where tissue specificity could play an important role.

4.4 Circadian Physiology Controlled by Noncanonical Clocks

Most circadian physiology is controlled by the circadian clock mechanisms described above, based upon feedback loops of transcription and translation. Very recently, however, another independent circadian mechanism was elucidated in red blood cells, which lack nuclei and therefore transcription. Although the mechanism of this clock remains entirely unknown, it is able to direct circadian oscillations of oxidation and reduction in both heme-containing proteins and peroxiredoxins, a highly conserved family of scavengers of peroxide produced by respiration (O'Neill and Reddy 2010). This clock mechanism appears to be independent of the known repertoire of clock proteins, and the range of physiology that it controls remains a mystery (for a review, see O'Neill et al. 2013).

5 Summary

Certainly, the discovery of peripheral oscillators in mammals qualifies as one of the major discoveries in circadian biology during the past twenty years. Through the vast amount of circadian biology that they control, these clocks doubtlessly play an important role in diurnal physiology, and specific disruption of clocks in peripheral tissues of laboratory mice can create a wide range of pathologies (Marcheva et al. 2013) – diabetes (Marcheva et al. 2010), atherosclerosis (Cheng et al. 2011), glucose intolerance (Lamia et al. 2008), and defects in renal and cardiac function (Ko et al. 2011; Zuber et al. 2009).

More important for human pathophysiology, because of the complex web of direct and indirect signals by which peripheral clocks are synchronized, it is likely that additional pathophysiology results from desynchrony between peripheral and central oscillators. In several studied instances, complex interactions between central clocks and peripheral ones maintain critical homeostasis – for example, in the case of glucose and insulin (Lamia et al. 2008; Marcheva et al. 2010). Since jet lag and shift work result in differential rates of clock adjustment in different tissues (Davidson et al. 2008), it is probable that some of the adverse pathologies associated with these conditions both in the laboratory and in the real world, such as metabolic syndrome (De Bacquer et al. 2009) and immune dysfunction (Castanon-Cervantes et al. 2010), could arise from conflict between peripheral and central clocks rather than from adverse effects of circadian phase shift per se. In this case, creative manipulation of peripheral clocks by synchronizing cues could provide possible therapeutic benefits. For example, reinforcement of circadian timing in peripheral tissues by meal timing has been shown to inhibit cancer growth by 40% in mice, irrespective of caloric intake (Li et al. 2010).

In this review, we have tried to separate and enumerate the various different mechanisms and entrainment signals for peripheral circadian clocks, as well as the physiology that they control. The resulting picture that emerges, though complex, is

likely far too simple. In reality, it is likely that clocks in different tissues interact in many different layers. For example, as explained above for nuclear-receptor-mediated physiology, many NR ligands are expressed in circadian fashion via circadian neuroendocrine control by the autonomous nervous system, but the synthesis of these steroid hormones depends upon autonomous circadian clocks in endocrine tissues. Circadian oscillations in hormone abundance program a circadian physiological response in target tissues, but clock components in these tissues then provide a further layer of circadian regulation. The physiological consequences of such networks are not yet fully understood but will doubtlessly furnish fascinating and medically relevant subjects of investigation in the future.

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